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IMMUNE RESPONSES AGAINST TUMORS CONTROLLED BY THE ACTIN CYTOSKELETON

LESSONS FROM PRIMARY IMMUNODEFICIENCIES

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Lessons from Primary Immunodeficiencies

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To my parents,
for giving me everything

There is nothing like looking, if you want to find something.
You certainly usually find something, if you look,
but it is not always quite the something you were after.

J.R.R Tolkien

ABSTRACT

The actin cytoskeleton and its regulators are vital for cellular processes such as cell motility and immune synapse formation. In this study, the aim was to dissect the role of two of those regulators, WASp and MKL1, in immune cells and their role in tumorigenesis. Additionally, we pharmacologically target an enzyme involved in fatty acid metabolism in monocytes and assess the impact on the actin cytoskeleton.

Deleterious mutations in WASp cause the Wiskott – Aldrich syndrome (WAS) and activating mutations cause X-linked neutropenia (XLN). Both immunodeficiencies are accompanied by a higher risk of developing malignancy. In **paper I** we investigated NK cell function when WASp is absent. We found that WASp-deficient NK cells were hyporesponsive to stimulation and failed to form immune synapses with tumor cells *in vitro* and to reject MHC Class I-deficient splenocytes *in vivo*. However, we observed no defect in lymphoma development or rejection in vivo. IL-2 is a cytokine known to stimulate NK cell cytotoxicity. When incubating NK cells with IL-2, we could rescue the functional defects of degranulation and cytokine secretion, as well as actin polarization to the synapse *in vitro*. Additionally, when injecting the IL-2 treated NK cells into WASp-deficient that have received MHC Class I-deficient splenocytes, we could also rescue the *in vivo* defect. Interestingly, we found that all the lymphomas we used in this study were capable of producing large amounts of IL-2 *in vivo*, which could be the reason that WASp-deficient NK cells were capable of handling the tumors similarly to wildtype. Therefore, we conclude that the malignancies observed in WAS are likely due to immune surveillance defects and can be circumvented by immunotherapy. In **paper II**, we dissect the role of activating WASp mutations in NK cell and T cell functionality in the context of tumor development. We found that NK cells and T cells from mice with activating mutations $WASp^{L272P}$ and $WASp^{I296T}$ were capable of responding to receptor stimulation as well as form immune synapses *in vitro*. $WASp^{L272P}$ mice could reject lymphomas to the same extent as wildtype and had higher rejection rates of MHC Class I-deficient splenocytes in vivo. Additionally, NK cells from $WASp^{L272P}$ and $WASp^{I296T}$ mice had lower amounts of KLRG1, an inhibitory receptor. NK cells from XLN patients with the L270P mutation were hyporesponsive to stimulation with tumor cells but not to PMA/Ionomycin and had decreased KLRG1 expression. Interestingly, the patients' T cells had increased amounts of KLRG1, high Granzyme B content and could respond to PMA/Ionomycin stimulation. There was also the appearance of a $CD4^{+}CD8^{low}$ population of T cells in the patients. Therefore, the malignancies observed in XLN are more likely due to increased cell intrinsic transformational capacity than defects in immunosurveillance. While the cytoplasmic role of WASp is extensively studied, both in general and in the context of this thesis, the nuclear role of WASp is less known. In **paper V**, we sought to determine that role. We show that WASp is indeed present in the nucleus and mediates active transcription. Additionally, we observed that WASp drives transcriptional events associated with T cell development and does this through its regulation of T cell-specific transcription factor TCF1. We also found that WASp interacted with TCF1 but had no direct binding to it; the proteins could instead form a macromolecular complex.

Paper III, focuses on the actin regulator MKL1 and attempts to dissect its role in the development of Hodgkin's lymphoma (HL). We investigated triplets with a deletion in the first intron of MKL1, two of which developed and were treated for HL. We found that the deletion induced increased expression of MKL1. In EBV-transformed B cells we generated from the triplets, we saw that the cells from the unaffected triplet (termed HL0) showed decreased aggregation, increased spreading and protrusion formation on slides, increased proliferation, and increased genomic instability. These phenotypic traits have all been connected to a more invasive cellular state. These findings, along with the increased expression of MKL1 in various lymphomas that we looked at, provide evidence for the link between MKL1 and lymphoma development.

The actin cytoskeleton can be hijacked by cancer cells to produce highly motile and invasive cells. In **paper IV** we used and inhibitor for the enzyme 15-lipoxygenase-1 (15-LOX-1) that mediates metabolism of fatty acids. We showed that the enzyme is necessary for the formation of podosomes in dendritic cells (DCs), which could contribute to invasion and metastasis. The DCs were also impaired in their migratory and endocytic capacity when treated with the inhibitor. T cell responses when using the inhibitor on DCs were only marginally affected.

POPULAR SCIENCE SUMMARIES

English

The immune system can be seen as a collection of organs, cells, and barriers that block pathogens such as bacteria and viruses from entering the body and causing disease. However, threats can arise in the body itself, causing diseases such as cancer. In this thesis, I take a look at several cells of the immune system and their behavior when proteins of the actin cytoskeleton are affected. The cytoskeleton, much like our own skeleton, helps the cell maintain its form, move, and perform other important cellular functions. It is very important in the elicitation of proper immune responses against, for example, cancer.

In papers I, II and IV we look at a protein called WASp and how it can affect the body's immune response against cancer. In **paper I**, the disease we use as a model to study immune cells is called the Wiskott – Aldrich Syndrome. It is a so-called immunodeficiency and it is caused by mutations that lead to the Wiskott – Aldrich Syndome protein (WASp in short) being produced less or not at all. One of the symptoms of WAS is an increased risk of developing cancer. WASp is a very important protein for the cytoskeleton of the cell. Perhaps most importantly for the cells we study here, WASp controls the release of small vesicles in specialized cells that can kill infected or cancerous cells. These cells are called Natural Killer (NK) cells. In a person with a normal immune system, NK cells are one of the first cell types that will start responding to a threat. In WAS patients, however, and as we show here in mice that lack WASp, their NK cells are not capable of responding to stimulation. But when the tumor cells that we inject in the mice produce a molecule called IL-2, the NK cells from the WASp deficient mice can respond normally to tumors. This molecule is very important for NK cell function and it is now in clinical trials for preventing WAS patients from getting cancer. In **paper II** we take a look at another genetic immunodeficiency called X-linked neutropenia (XLN). It is also caused by mutations in WASp but the protein is instead overactive rather than absent. This causes a whole other set of symptoms in patients but still presents a heightened risk of developing cancer. We again took a look at the NK cells from mice that were engineered to express the mutant form of WASp. In the case of mice with XLN, their NK cells were remarkably capable of responding to stimulation and killing cancer cells, even more than in a normal mouse. We also got some material from these very rare patients with XLN and saw the same in their NK cells. In conclusion, NK cells from XLN mice might be a little bit too good. NK cells that are overactive are known to turn on the body's own cells causing autoimmune symptoms. In **paper V**, we look at the role of WASp in the nucleus, the brain of the cell. We saw that WASp mediates the decision of what an immune cell should become. WASp is important for a cell to properly turn into a T cell. T cells are also important in the fight against tumors. When WASp is either absent or overactive, we saw that transcription of specific proteins was altered. This could contribute to the pathogenesis of the WAS and XLN syndromes. We therefore identified a new role for WASp in the nucleus in the development and functionality of T cells.

In papers III and IV, we look at how the development of certain cancers is influenced by a specific protein and at a drug that influences the way immune cells respond to inflammation. **Paper III** involves another actin cytoskeleton regulator protein, called MKL1. MKL1 has been shown to be mutated in several cancer types. Here we looked at a specific type of blood cancer, called Hodgkin's lymphoma, and how MKL1 could be involved in its development. We got blood samples from triplets that had a deletion in their MKL1 gene and 2 of the 3 brothers had developed Hodgkin's lymphoma. Interestingly, this deletion of MKL1 caused the protein to be overexpressed in the triplets' immune cells. We could show that this overexpression of MKL1 caused their cells to behave in a way very reminiscent of cancer cell behavior. They would grow much faster, form extensions of their cytoskeleton capable of mediating metastasis and had problems keeping their DNA intact. We therefore concluded that mutations in MKL1 could be an underlying cause for Hodgkin's lymphoma. In **paper IV** we used a drug against an enzyme that is very important in the regulation of various immune responses. This drug inhibited the enzyme's function in immune cells called dendritic cells and caused the formation of fewer protrusions, indicative of highly motile cells, like cancer cells. Therefore, this drug could be a good solution for treatment of immune diseases, such as cancer.

Svenska

Immunsystemet kan ses som en samling av organ, celler och barriärer som blockerar patogener som bakterier och virus från att komma in i kroppen och orsaka sjukdom. Men hot kan uppstå i själva kroppen, vilket orsakar sjukdomar som cancer. I denna avhandling tittar jag på flera celler i immunsystemet och deras beteende när proteiner av aktincytoskelet påverkas. Cytoskelettet, liknande vårt eget skelett, hjälper cellen att behålla sin form, röra sig och utföra andra viktiga cellulära funktioner. Det är mycket viktigt vid framkallandet av ett korrekt immunförsvar mot exempelvis cancer.

I artiklar I, II och IV tittar vi på ett protein som heter WASp och hur det kan påverka kroppens immunförsvar mot cancer. I **artikel I** använder vi en sjukdom som heter Wiskott – Aldrich syndromet som modell för att studera immunceller. Det är en så kallad immunbrist-sjukdom och orsakas av mutationer som leder till att Wiskott – Aldrich Syndome proteinet (WASp i korthet) produceras mindre eller inte alls. Ett av symtomen av WAS är en ökad risk att utveckla cancer. WASp är ett mycket viktigt protein för cytoskelettet i cellen. För de celler vi studerat är WASp viktigt för att det kontrollerar frisättningen av små vesiklar i specialiserade celler som kan döda infekterade celler eller cancerceller. Dessa celler heter Natural Killer (NK) celler. Hos en person med normalt immunsystem är NK cellerna en av de första celltyperna som börjar reagera på ett hot. I WAS patienter, och som vi visar här hos möss som saknar WASp, kan deras NK celler inte reagera på stimulering. Men när de tumörceller som vi injicerar i mössen producerar en molekyl som heter IL-2, kan NK cellerna från de mössen utan WASp svara normalt på tumörer. Denna molekyl är mycket viktig för NK cellers funktionalitet och finns nu i kliniska tester för att förhindra WAS patienter från att få cancer. I **artikel II** tittar vi på en annan genetisk immunbrist som heter X-linked

neutropenia (XLN). Den orsakas också av mutationer i WASp men i detta fall är proteinet överaktivt i motsats till frånvarande. Detta orsakar en hel annan uppsättning symtom i patienten men XLN patienter har också högre risk att utveckla cancer. Här tog vi en titt på NK cellerna från möss som konstruerades för att uttrycka den mutanta formen av WASp. I möss med XLN är deras NK celler anmärkningsvärt kapabla att reagera på stimulering och att döda cancerceller, ännu mer än i en vanlig mus. Vi fick också material från dessa mycket sällsynta patienter med XLN och såg samma i deras NK celler. Sammanfattningsvis kan det vara så att NK celler från XLN möss är lite för bra. NK celler som är överaktiva kan attackera kroppens egna celler och orsaka autoimmuna symptom. I **artikel V** tittar vi på rollen av WASp i kärnan, cellens hjärna. Vi såg att WASp medierar beslutet om vad en immun cell ska bli. WASp är viktigt för att en cell ska omvandlas till en T cell. T celler är också viktiga i kampen mot tumörer. När WASp är antingen frånvarande eller överaktivt såg vi att transkriptionen av specifika proteiner förändras. Detta kan bidra till patogenasen av WAS och XLN syndromen. Vi identifierade därför en ny roll för WASp i kärnan i utvecklingen och funktionaliteten av T celler.

I artiklar III och IV tittar vi på hur utvecklingen av vissa cancerformer påverkas av ett specifikt protein och på ett läkemedel som påverkar hur immunceller svarar mot inflammation. **Artikel III** innefattar ett annat protein som reglerar aktincytoskelettet, kallat MKL1. MKL1 har visats vara muterat i flera cancertyper. Här tittar vi på en specifik typ av blodcancer, kallad Hodgkins lymfom, och hur MKL1 kan vara inblandad i utvecklingen av det. Vi fick blodprover från trillingar som hade en deletion i deras MKL1 gen och 2 av de 3 bröderna har utvecklat Hodgkins lymfom. Intressant är att denna deletion av MKL1 orsakade att proteinet överuttrycktes i trillingarnas immunceller. Vi kunde visa att överuttrycket av MKL1 orsakade deras celler att uppträda på ett sätt som påminner om beteendet av cancer celler. De kunde växa mycket snabbare, bilda förlängningar av deras cytoskelett som skulle kunna mediera metastaser och hade problem med att hålla sitt DNA intakt. Vi drog därför slutsatsen att mutationer i MKL1 kan vara en bakomliggande orsak till Hodgkins lymfom. I **artikel IV** använde vi ett läkemedel mot ett enzym som är mycket viktigt vid reglering av olika immunförsvar. Detta läkemedel inhiberade enzymets funktion i immunceller som kallas dendritiska celler och orsakade bildandet av färre utsprång, vilket indikerar rörliga celler, som cancerceller. Därför kan detta läkemedel vara en bra lösning för behandling av immunsjukdomar, såsom cancer.

Ελληνικά

Το ανοσοποιητικό σύστημα μπορεί να θεωρηθεί ως μια συλλογή οργάνων, κυττάρων και φραγμών που εμποδίζουν την είσοδο παθογόνων οργανισμών, όπως βακτήρια και ιούς, στο σώμα και την πρόκληση ασθενειών. Ωστόσο, απειλές μπορεί να προκύψουν στο ίδιο το σώμα, προκαλώντας ασθένειες όπως ο καρκίνος. Σε αυτή τη διατριβή, ρίχνω μια ματιά σε διάφορα κύτταρα του ανοσοποιητικού συστήματος και τη συμπεριφορά τους όταν επηρεάζονται πρωτεΐνες του κυτταροσκελετού ακτίνης. Ο κυτταροσκελετός, όπως και ο δικός μας σκελετός, βοηθάει το κύτταρο να διατηρεί τη μορφή του, να κινείται και να εκτελεί

άλλες σημαντικές κυτταρικές λειτουργίες. Είναι πολύ σημαντικός για την πρόκληση σωστών ανοσοαποκρίσεων έναντι, για παράδειγμα, του καρκίνου.

Στα άρθρα I, II και IV εξετάζουμε μια πρωτεΐνη που ονομάζεται WASp και πως μπορεί να επηρεάσει την ανοσολογική απόκριση του οργανισμού ενάντια στον καρκίνο. Στο **άρθρο I**, η ασθένεια που χρησιμοποιούμε ως μοντέλο για τη μελέτη κυττάρων του ανοσοποιητικού συστήματος ονομάζεται σύνδρομο Wiskott – Aldrich. Το σύνδρομο αυτό είναι μια αποκαλούμενη ανοσοανεπάρκεια και προκαλείται από μεταλλάξεις που οδηγούν στο να παράγεται λιγότερο ή καθόλου η πρωτεΐνη Wiskott – Aldrich Syndome (WASp εν συντομία). Ένα από τα συμπτώματα του WAS είναι ο αυξημένος κίνδυνος ανάπτυξης καρκίνου. Η πρωτεΐνη WASp είναι πολύ σημαντική για τον κυτταροσκελετό του κυττάρου. Για τα κύτταρα που μελετάμε εδώ, υψίστης σημασίας αποτελεί το γεγονός ότι η WASp ελέγχει την απελευθέρωση μικρών κυστιδίων από εξειδικευμένα κύτταρα που μπορούν να σκοτώσουν μολυσμένα ή καρκινικά κύτταρα. Αυτά τα κύτταρα ονομάζονται Natural Killer (NK) ή κύτταρα Φυσικοί Φονιάδες στα ελληνικά. Σε άτομα με φυσιολογικό ανοσοποιητικό σύστημα, τα κύτταρα NK είναι ένας από τους πρώτους τύπους κυττάρων που θα αρχίσουν να ανταποκρίνονται σε απειλή. Ωστόσο, σε ασθενείς με WAS, και όπως δείχνουμε εδώ σε ποντίκια που δεν έχουν WASp, τα NK κύτταρά τους δεν είναι ικανά να ανταποκριθούν σε σημάδια ενεργοποίησης. Αλλά όταν τα καρκινικά κύτταρα που εισάγουμε στα ποντίκια παράγουν ένα μόριο που ονομάζεται IL-2, τα NK κύτταρα στα ποντίκια με ανεπάρκεια στη WASp μπορούν να αποκριθούν κανονικά σε όγκους. Αυτό το μόριο είναι πολύ σημαντικό για τη λειτουργία των NK κυττάρων και τώρα βρίσκεται σε κλινικές δοκιμές για την πρόληψη του καρκίνου σε ασθενείς με WAS. Στο **άρθρο II** εξετάσαμε μια άλλη γενετική ανοσοανεπάρκεια που ονομάζεται X-linked neutropenia (XLN), ή ουδετεροπενία που συνδέεται με το χρωμόσωμα Χ στα ελληνικά. Προκαλείται επίσης από μεταλλάξεις στη WASp αλλά εδώ η πρωτεΐνη είναι υπερδραστήρια σε αντίθεση με απούσα. Αυτό προκαλεί ένα σύνολο διαφορετικών συμπτωμάτων στον ασθενή αλλά οι ασθενείς με XLN έχουν επίσης υψηλότερο κίνδυνο ανάπτυξης καρκίνου. Εξετάσαμε και πάλι τα κύτταρα NK από ποντίκια που κατασκευάστηκαν για να εκφράσουν τη μεταλλαγμένη μορφή της WASp. Στην περίπτωση των ποντικιών με XLN, τα NK κύτταρα τους είναι αξιοσημείωτα ικανά να αποκρίνονται και να σκοτώνουν καρκινικά κύτταρα, ακόμη περισσότερο από ότι σε ένα κανονικό ποντίκι. Έχουμε επίσης υλικό από αυτούς τους πολύ σπάνιους ασθενείς με XLN και είδαμε το ίδιο και στα δικά τους NK κύτταρα. Εν κατακλείδι, τα NK κύτταρα από XLN ποντίκια μπορεί να είναι λίγο καλύτερα από ότι θα έπρεπε. NK κύτταρα που είναι υπερδραστήρια είναι γνωστό ότι επιτίθενται στα κύτταρα του ίδιου του σώματος, προκαλώντας αυτοάνοσα συμπτώματα. Στο **άρθρο V**, εξετάσαμε το ρόλο της WASp στον πυρήνα, τον εγκέφαλο του κυττάρου. Είδαμε ότι η WASp μεσολαβεί στην απόφαση για το τι θα γίνει ένα ανοσοποιητικό κύτταρο. Η WASp είναι σημαντική για ένα κύτταρο να μετατραπεί σωστά σε ένα Τ λυμφοκύτταρο. Τα Τ κύτταρα είναι επίσης σημαντικά στην καταπολέμηση των όγκων. Είδαμε ότι όταν η WASp είτε απουσιάζει είτε είναι υπερδραστήρια, μεταβάλλεται η μεταγραφή συγκεκριμένων πρωτεϊνών. Αυτό θα μπορούσε να συμβάλει στην παθογένεση των συνδρόμων WAS και XLN. Επομένως, προσδιορίσαμε

έναν νέο ρόλο για την WASp στον πυρήνα και την ανάπτυξη και λειτουργικότητα των Τ κυττάρων.

Στα άρθρα ΙΙΙ και IV, εξετάσαμε πώς η ανάπτυξη ορισμένων καρκίνων επηρεάζεται από μια συγκεκριμένη πρωτεΐνη και πως ένα φάρμακο επηρεάζει τον τρόπο με τον οποίο τα κύτταρα του ανοσοποιητικού συστήματος ανταποκρίνονται στη φλεγμονή. Το **άρθρο ΙΙΙ** περιλαμβάνει μια άλλη ρυθμιστική πρωτεΐνη του κυτταροσκελετού ακτίνης, που ονομάζεται MKL1. Η MKL1 μεταλλάσσεται σε διάφορους τύπους καρκίνου. Εδώ εξετάσαμε έναν συγκεκριμένο τύπο καρκίνου του αίματος, που ονομάζεται λέμφωμα Hodgkin, και πώς η MKL1 θα μπορούσε να συμμετάσχει στην ανάπτυξή του. Πήραμε δείγματα αίματος από τρίδυμα που είχαν διαγραφή στο γονίδιο MKL1 και 2 από τα 3 αδέλφια έχουν αναπτύξει λέμφωμα Hodgkin. Αυτή η διαγραφή του γονιδίου MKL1 προκάλεσε την πρωτεΐνη να υπερεκφράζεται στα ανοσοκύτταρα των τριδύμων. Μπορούσαμε να δείξουμε ότι αυτή η υπερέκφραση της MKL1 προκάλεσε τα κύτταρα τους να συμπεριφέρονται με τρόπο που θυμίζει πολύ τη συμπεριφορά καρκινικών κυττάρων. Μπορούσαν να πολλαπλασιαστούν πολύ ταχύτερα, να προκαλέσουν επεκτάσεις του κυτταροσκελετού τους που θα μπορούσαν να συμβάλουν στην μετάσταση, και είχαν προβλήματα στο να διατηρήσουν το DNA τους άθικτο. Συνεπώς, καταλήξαμε στο συμπέρασμα ότι οι μεταλλάξεις στο MKL1 θα μπορούσαν να αποτελέσουν υποκείμενη αιτία για το λέμφωμα Hodgkin. Στο **άρθρο IV** χρησιμοποιήσαμε ένα φάρμακο εναντίον ενός ενζύμου που είναι πολύ σημαντικό στη ρύθμιση διαφόρων ανοσολογικών αποκρίσεων. Αυτό το φάρμακο αναστέλλει τη λειτουργία του ενζύμου σε κύτταρα του ανοσοποιητικού συστήματος που ονομάζονται δενδριτικά κύτταρα και προκάλεσε το σχηματισμό λιγότερων προεξοχών που είναι ενδεικτικές εξαιρετικά κινητικών κυττάρων, όπως τα καρκινικά κύτταρα. Ως εκ τούτου, αυτό το φάρμακο θα μπορούσε να είναι μια καλή λύση για τη θεραπεία ανοσολογικών ασθενειών, όπως ο καρκίνος.

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II. **Kritikou JS**, Rentouli S, Wagner AK, Keszei M, Brauner H, Snapper SB, Kärre K, Vandenberghe P, Orange JS, and Westerberg LS.
NK cells with constitutively active WASp display hyperactivity and increased tumor cell killing.
Manuscript

III. Sendel A*, Record J*, **Kritikou JS**, Kuznetsov NV, Brauner H, He M, Nagy N, Griseti E, Liu C, Andersson J, Claesson HE, Winqvist O, Burns SO, Björkholm M, Westerberg LS.
*denotes equal contribution
An intronic deletion in MKL1 is associated with hyperproliferation of B cells in triplets with Hodgkin lymphoma
Manuscript

IV. Han H, Liang X, Ekberg M, **Kritikou JS**, Brunnström Å, Pelcman B, Matl M, Miao X, Andersson M, Yuan X, Schain F, Parvin S, Melin E, Sjöberg J, Xu D, Westerberg LS, Björkholm M, Claesson HE.
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V. Kuznetsov NV, Almuzzaini B, **Kritikou JS**, Baptista MA, Oliveira M, Keszei M, Snapper SB, Percipalle P, Westerberg LS.
Nuclear WASp co-regulates TCF1-mediated transcription in T cells
Manuscript – accepted Genome Medicine

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Exercise and BDNF reduce Aβ production by enhancing α-secretase processing of APP.
J Neurochem. 2017 Jul;142(2):286-296.

CONTENTS

LIST OF ABBREVIATIONS

ABP	Actin Binding Protein
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADP	Adenosine Diphosphate
AIRE	Autoimmune Regulator
APC	Antigen Presenting cell
Arp	Actin related protein
ATP	Adenosine Triphosphate
BC	Before Christ
BCR	B cell Receptor
CCR7	C-C chemokine Receptor type 7
CD	Cluster of Differentiation
Cdc42	Cell division control protein 42
CLP	Common Lymphoid Progenitor
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein-4
DC	Dendritic Cell
DLC1	Deleted in Liver Cancer 1
DN	Double Negative
DNA	Deoxyribonucleic Acid
DNAM-1	DNAX Accessory Molecule-1
DP	Double Positive
EBV	Epstein-Barr virus
ECM	Extracellular Matrix
Flt3L	FMS-like Tyrosine kinase 3 Ligand
FO	Follicular
GAP	GTPase-activating or GTPase-accelerating proteins
G-CSF	Granulocyte – Colony Stimulating Factor
GDP	Guanosine Diphosphate
GEF	Guanine nucleotide Exchange Factor
GM-CSF	Granulocyte-Macrophage – Colony-stimulating Factor
GTP	Guanosine Triphosphate
GvHD	Graft-vs-Host Disease
GvL	Graft-vs-Leukemia
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cell Transplantation
IFN	Interferon
IL	Interleukin
ILC	Innate Lymphoid cell
IS	Immunological or Immune Synapse
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif

ITSM	Immunoreceptor Tyrosine-based Switch Motif
KIR	Killer-cell Immunoglobulin-like Receptor
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LAG3	Lymphocyte Activation Gene 3
LAK	Lymphokine Activated Killer
LCL	Lymphoblastoid cell line
LFA-1	Lymphocyte Function-associated Antigen-1
MHC	Major Histocompatibility Complex
MKL1	Megakaryoblastic Leukemia (Translocation) 1
MTOC	Microtubule-organizing Center
MZ	Marginal Zone
NCR	Naturaral Cytotoxicity Receptors
NK	Natural Killer cell
NKP	NK cell Precursor
NKT	Natural Killer T cell
PBMC	Peripheral Blood Mononuclear Cell
PD-1	Programmed cell Death-1
PID	Primary Immune Deficiency
PIP(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
PRR	Pattern Recognition Receptor
SCID	Severe Combined Immunodeficiency
SH	Src Homology region
SHIP	SH-containing Inositol Phosphatase
SHP	SH-containing Phosphatase
SLAM	Signaling Lymphocytic Activating Molecule
SRF	Serum Response Factor
Tc or CTL	Cytotoxic T lymphocyte
TCF	T Cell Facror
TCR	T cell Receptor
TEC	Thymic Epithelial cell
Th	T helper cell
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAIL	TNF-related Apoptosis-inducing Ligand
WAS	Wiskott – Aldrich Syndrome
WASp	Wiskott – Aldrich Syndrome protein
WIP	WASp Interacting Protein
XLN	X-linked Neutropenia

1 INTRODUCTION

1.1 PRELUDE

The main components of this thesis are the actin cytoskeleton, tumorigenesis and, perhaps most importantly, lessons learned from primary immunodeficiencies. While writing about these concepts, I find myself unable to neglect mentioning the story of David Vetter. David was born in 1971 and is, most affectionately, known as "the boy in the bubble". He suffered from a disease called Severe Combined Immune Deficiency (SCID), which caused a complete absence of B and T cells. Being at a major risk for life-threatening infections, David lived in protected environments to maintain relatively germ-free surroundings at the Texas Children's Hospital. The only treatment option at the time of David's birth was bone marrow transplantation. After an exhaustive search to find a matching donor, David finally underwent surgery in 1984. Sadly, four months after his transplantation, David passed away from lymphoma caused by the Epstein-Barr virus (EBV).

In my view, science has come a remarkably long way since then. There are diagnostic tools readily available for use in newborns and treatment options, such as gene therapy, that were long considered science fiction. This thesis attempts to discuss all of these breakthroughs and, hopefully, contribute to the information needed to cure, not only immunodeficiencies, but also other life-threatening immune disorders.

1.2 THE IMMUNE SYSTEM

Some of the first documented references of the immune system and immunity came from Hippocrates: "*Natural forces within us are the true healers of disease*" c. 460-370 BC, and Thucydides: "*Though many lay unburied, birds and beasts would not touch them, or died after tasting them… [But] those who had recovered from the disease had now no fear for themselves; for the same man was never attacked twice, never at least fatally.*" History of the Peloponnesian War, 431–428 BC.

The immune system is a defense system comprising of numerous biological structures, processes, cells, and molecules that protects organisms against disease. The fact that we are constantly exposed to microorganisms but only rarely develop disease can be attributed to a well-functioning immune system. The functions of the immune system are (mainly) exerted by immune cells. All immune cells derive from precursors in the bone marrow, called hematopoietic stem cells, through a process called hematopoiesis[A]. The immune system is considered as having two branches; the innate immune system and the adaptive immune system. They are interconnected by various mediators and cells but have several distinct characteristics.

[A] Hematopoiesis: From Greek αἷμα "blood" and ποιεῖν "make", formation of the cellular components of blood.

1.2.1 The innate immune system

The innate immune system is evolutionarily older than the adaptive and is found in both vertebrates and invertebrates. Recognition of pathogens by the innate immune system relies on a set of germline encoded receptors on the surface of innate immune cells. The innate immune response occurs within minutes or hours of infection. The first line of defense is molecular; various antimicrobial peptides and enzymes as well as the complement system, a collection of plasma proteins, target the invading pathogens for lysis or phagocytosis by cells of the innate immune system. The second phase of the response involves receptors on the surface of cells such as macrophages that recognize the pathogen as potentially dangerous. These receptors are termed pattern recognition receptors (PRRs) and can recognize three danger signals: non-self, missing-self, and altered-self[1]. Two other textbook characteristics of the innate immune system are the lack of antigen specificity and of immunological memory. However, the nonexistence of memory in innate responses has begun to be disputed[2–4]. There have been reports on plants and invertebrates that lack adaptive immune systems being protected against secondary infections. Mammals have also been shown to have the ability to build cross-protection against secondary infections independently of T and B cells. Moreover, innate immune cells such as NK cells and monocytes can display adaptive characteristics. NK cells, for example, have been shown to respond better to secondary stimulation and a pool of long-lived, self-renewing "memory NK cells" has been detected in mice[5–8].

The cells that perform the effector functions of the innate immune system belong to both the myeloid and lymphoid lineages. Myeloid cells include mast cells, granulocytes (neutrophils, basophils, and eosinophils), and monocytes. The latter can further differentiate into macrophages[B] and dendritic cells (DCs). The lymphoid compartment of the innate immune system includes Natural Killer (NK), Natural Killer T (NKT) cells, and γδ T cells. For the purposes of this thesis, NK cells and dendritic cells will be analyzed further.

1.2.1.1 Natural Killer cells

Natural Killer (NK) cells[C], as their name suggests, are potent killers of virally infected or transformed cells and do so without prior sensitization. Recently, it became clear they are part of a larger innate lymphoid cell (ILC) group[9–12]. Like NK cells, ILCs lack antigen-specific receptors and only have germline encoded ones. ILCs are predominantly found in tissues that are in close proximity to commensal bacteria and other microorganisms, like the gut, skin, blood, and lungs. They are potent producers of cytokines upon stimulation and are important for tissue homeostasis and development. Analogy can be made to the T cell lineage; NK cells are the cytotoxic cells and the ILCs are the helper cells of the innate immune system.

[B] Macrophage, from Greek μακρός "large" and φαγεῖν "eat". Macrophages are known as the "big eaters" of the immune system. The discovery of phagocytes is credited to Élie Metchnikoff in 1882.
[C] The name "Natural Killer" was originally proposed by Eva Klein.

NK cells were first discovered in the 1970s when researchers were in search of tumor neo-antigens that could activate T cell responses[13,14]. They were initially considered to be background noise and *in vitro* artifacts in T cell cytotoxicity assays from studies performed both with mouse and human cells[15]. That turned out not to be the case and the new cell type was named "Natural Killer" cell, since it could mount a rapid, naturally-occurring, cytotoxic response against YAC-1 Moloney leukemia cells[13]. This cytotoxicity was shown to not be mediated by T cells, B cells, or macrophages nor did it involve antibody dependent cell mediated cytotoxicity (ADCC)[13,14]. Therefore, it seemed like this was indeed a new cytotoxic cell type. NK cells would later be found in mice starting at 3 weeks of age and their numbers were most abundant in the spleen but they were also present in lymph nodes and the bone marrow[16]. Very soon after the discovery of NK cells, researchers believed in their potency against tumor cells and in the control of viral infections. What was elusive though was the mechanism by which NK cells specifically recognize these cells and mount a cytotoxic response, while leaving normal cells intact. Clues came from observations made in mice that NK cell cytotoxicity is controlled by genes in the H-2 locus and that NK cells were the cells responsible for "hybrid resistance"[17]. Hybrid resistance is a phenomenon where F1 hybrid mice rejected allogeneic but also parental hematopoietic grafts. But it was first in the 1980s when the "missing-self hypothesis" was formed and the lack of MHC Class I on the surface of target cells was found to be an extremely potent activating signal for NK cells[18]. Today, we know that apart from their role in direct elimination of tumor cells[19–21] and being vital in the control of multiple infections[22,23], NK cells have additional functions including maintaining the homeostasis in the lymphoid compartment, protecting against auto-immunity[24], and in reproduction and pregnancy[25–27].

NK cells develop in the bone marrow from the common lymphoid progenitor (CLP) that can also give rise to B and T cells and some DC subsets. The CLP goes on to become the pre-NK precursor (pre-NKP) that has lost the ability to become cells of the other lymphoid lineages[28,29]. The pre-NKPs differentiate into NKPs that now start to express the interleukin (IL)-15 receptor complex[28,29] and NKPs further differentiate into immature NK cells that start to express NK1.1 and NKG2D but are not yet functional[30,31]. Thereafter, immature NK cells egress from the bone marrow and start to express DX5 and Ly49 receptors[30–32]. NK cells in the periphery can further mature into subset characterized by differential expression of CD11b and CD27[33,34]. Several cytokines and transcription factors are important for NK cell development, survival and homeostasis. Transcription factors Id2, T-bet, and Eomes[28,35,36] are vital for NK cell differentiation and cytokines, such as IL-2 and IL-15[37,38], are important for their survival, differentiation, and proliferation. Interestingly, IL-15 has been shown to be trans-presented by DCs[39]. In order for NK cells to be able to distinguish self from non-self they first need to be educated on what constitutes self. Inhibitory receptors that recognize MHC Class I are not only important for inhibitory signaling in NK cell effector responses, but also for tuning NK cell responsiveness prior to the effector phase[40–46]. As a result of the education process, only NK cells that express inhibitory receptors for self-MHC Class I, and therefore can be inhibited by normal cells, will gain the capacity of effector functions. These

same NK cells will be able to recognize MHC Class I downregulation on the surface of, for example, tumor cells and respond with cytotoxicity against them.

Apart from the education process, NK cell effector functions are controlled by signals via two groups of germline encoded receptors, the activating and the inhibitory receptors, as well as many co-stimulatory and adhesion receptors. The signals from all engaged receptors have to be integrated and the functional outcome of that is the decision of NK cell responsiveness vs. non-responsiveness[47–49].

Activating receptors signal via immunoreceptor tyrosine-based activation motifs (ITAMs) that are either a part of the receptor itself or of associated adaptor proteins. After ligand binding, ITAMs become phosphorylated by Src kinases[50] and downstream signaling is initiated. Activating receptors found on NK cells include: *CD16* (FcγRIII) binds the Fc part of antibodies and induces ADCC[51]. *NK1.1* is a C-type lectin encoded by the NK gene complex (NKC) and is only expressed on NK cells in mice of the C57Bl/6 strain[52]. *NKp46* belongs to a receptor group called natural cytotoxicity receptors (NCRs) and is the only member of the group found in mice. In humans, additional members are NKp30 and NKp44[47]. They recognize ligands on the surface of stressed cells[53] and viral hemagglutinins[54]. *NKG2D* is a C-type lectin, found on NK cells and some subsets of T cells, that recognizes ligands upregulated on stressed cells[55]. Ligands for NKG2D are Rae1, Mult1, and H60 in mice and MICA/B, and ULBP1-6 in humans, all of which share similarities with MHC Class I molecules[55]. Tumor cells initially expressing NKG2D ligands try to shed them, but that has been shown to cause enhanced anti-tumor NK cell responses[56]. *DNAM-1* functions both as an activating receptor and an adhesion molecule by binding its ligands CD155 and CD112[57]. DNAM-1 ligands are also upregulated on stressed cells and DNAM-1 has been linked to maturation and education of NK cells[58,59]. Most of the members of the Ly49 Receptor family are inhibitory but there are some Ly49Rs that mediate activating signaling. *Ly49D* recognizes H2-D^d, as does inhibitory receptor Ly49A that has a higher affinity for it[60]. *Ly49H* can recognize the m157 of cytomegalovirus (CMV) in mice and is responsible for the genetic resistance of some mouse strains to the virus[61,62].

Inhibitory receptors signal through immunoreceptor tyrosine-based inhibition motifs (ITIMs), which when phosphorylated recruit phosphatases to inhibit signaling that promotes NK cell activation. Src homology region 2 (SH2) domain-containing phosphatase 1 (SHP-1), SHP-2, and SH2 domain-containing inositol-5 phosphatase 1 (SHIP-1) are some of the phosphatases that can be utilized for inhibitory signaling[63]. The phosphatase recruited depends on which receptor is engaged. Inhibitory receptors include receptors that recognize MHC Class I, the Ly49R family in mice and the KIR family in humans, but also receptors that have other ligands instead of MHC Class I. The murine *Ly49Rs* are members of the C-type lectin-like family and are also encoded by the NKC. There are 8-18 different Ly49 genes, depending on the mouse strain, and they have extensive allelic polymorphisms[64–66]. In the C57Bl/6 mouse strain, the Ly49 locus codes for 10 receptors, 8 of which are inhibitory: Ly49A, B, C, E, F, G_2, I and J. They all recognize MHC Class I molecules but the

recognition requires a peptide to be bound in the MHC Class I cleft, in order for the correct conformational change to be obtained[67]. The *KIRs* found in humans make up a family of 15 members. They are Ig-like molecules containing two (KIR2D) or three (KIR3D) Ig-like domains. Their ITIM enables the recruitment of SHP-1 and inhibition of activating signaling. Inhibitory KIRs recognize HLA-C1, C2 and Bw4. The KIR repertoire differs between individuals but also on NK cells within one individual[40]. The *CD94/NKG2A* heterodimer, also encoded by the NKC, is expressed in both mice and humans. It interacts with the non-classical MHC Class I molecules Qa-I^b in mice and HLA-E in humans[68,69]. There are also inhibitory NK cell receptors whose ligands are not MHC Class I molecules. SLAM family receptors are expressed on cells of the hematopoietic lineage and are usually self-ligands. One SLAM receptor that is not a self-ligand and is expressed on NK cells is *2B4*, which binds to CD48[70]. 2B4 has four tyrosine-based switch motifs (ITSMs), which recruits adaptor proteins including SAP. SAP mediates NK cell cytotoxicity but in the absence of SAP, the ITSMs of 2B4 bind to phosphatases, which induces inhibitory signaling[71]. *KLRG1* is an adhesion molecule that recognizes E-, N-, and R-cadherins on the surface of epithelial and endothelial cells, and some DCs[72–74]. Cadherins have also been found to be downregulated on tumor cells[72]. KLRG1 is not only expressed on NK cells but also on activated and memory T cells[75,76]. On NK cells KLRG1 has been associated with education[77] and on $CD8^+$ T cells with exhaustion[78]. However, KLRG1 is dispensable for both NK cell and $CD8^+$ T cell development and function, as seen in KLRG1-deficient mice[79,80]. KLRG1 will be discussed further in the results section.

NK cell are rapid responders to activating stimuli due to pre-formed lytic granules that mediate their cytotoxic responses[81]. These NK cell granules contain a number of proteins, including a pore-forming protein termed perforin[82–84] and a family of serine proteases called granzymes[85,86]. Perforin causes osmotic damage due to its binding of phosphorylcholine groups, polymerization and subsequent pore formation in the lipid bilayer of the target cell[87]. Granzymes synergize with perforin to trigger apoptosis in the target cell. Granzyme B, for example, triggers apoptosis via critical target cell caspases[87]. Death can also be induced in the target cell by signaling via FasL and TRAIL[88]. NK cells also secrete cytokines, such as Interferon (IFN)γ and tumor necrosis factor (TNF)α, as well as chemokines and growth factors, rapidly upon activation[89]. These can in turn activate other cells of the immune system like those of the adaptive immunity[89].

For NK cells to exert their cytotoxic functions they need to form contacts with their target cells. Granules are released into the target cell via the contact area between the two cells, termed the *immunological synapse* (IS)[90–94]. The IS was originally defined in the late 1990s[95,96], as the crucial junction between a T cell and an APC, which enables T cell receptors to interact with MHC molecules. NK cells have been shown to form ISs with target cells in a regulated, stepwise manner[97–99]. Upon initial encounter with the target cell, NK cells undergo brief, exploratory interactions[100]. Signals through activating receptors strengthen this interaction and, among others, integrin LFA-1 becomes activated and mediates firm adhesion to the target cell[101]. This initializes the first steps of IS formation, including protein tyrosine

kinase activation, PIP(4,5)P2 generation, F-actin reorganization[102–104], and arrest of migration[105]. Polarization of the microtubule organizing center (MTOC) and lytic granules to the IS are also required for NK cell cytotoxicity. However, prior to polarization, lytic granules rapidly move along microtubules and converge upon the MTOC, which precedes NK cell commitment to cytotoxicity[106,107]. The effector phase of the IS formation is characterized by a commitment to cytotoxicity. After the commitment to cytotoxicity has been made, a *de novo* F-actin polymerization and reorganization occurs, largely dependent on members of the Wiskott – Aldrich syndrome protein family[108], which will be discussed later. The requirement for F-actin also entails permitting granules to be released, which occurs in a pervasive actin network at the IS[109,110]. This involves proteins such as cofilin, coronin 1A, Myosin IIA and others[111–115]. NK cell lytic granules have been found to use at least two distinct modes of fusion: Complete fusion where granule content is completely discharged and the contents diffuse rapidly at the plasma membrane, and incomplete fusion, whereby formation of a transient fusion pore at the plasma membrane is accompanied by release of some but retention of most of the granules[116]. The latter may promote efficient recycling of lytic granule membrane after the release of cytotoxic effector molecules[116]. The last stage of the IS is the termination stage. During this stage, NK cells appear relatively inactive and have to initiate a termination of their response. One possible mechanism is via perforin-induced exposure of phosphatidylserine that binds to NK cells and signals to terminate the response[117]. Additionally, receptor downregulation has been shown, for example for CD16 and NKG2D[118,119]. The final stage of cytotoxicity is defined by detachment from the target cell, the signal for which arises from the dying target cell and is caspase dependent[120]. Failure at the detachment stage can cause severe pro-inflammatory cytokine secretion and is therefore also a tightly regulated step[120]. After successful elimination of a target cell, NK cells can go on to kill additional target cells in a process called "serial killing"[121].

1.2.1.2 Dendritic cells

Dendritic cells (DCs)[p] were also discovered in the 1970s[122–126] and Ralph Steinman was awarded the Nobel Prize in Physiology/Medicine for his achievement, albeit they were first described by Paul Langerhans in the late 19th century. DCs constitute a heterogeneous group of cells that are highly specialized in antigen presenting[127,128]. They were initially thought to be part of the myeloid lineage but it is now evident that they can derive from lymphoid progenitors as well[129,130]. DCs exit the bone marrow as immature cells and constantly capture particles from their environment and scan for potential threats. They express a wide range of PRRs including Toll-like receptors (TLRs) and scavenger receptors. There are two major subsets of DCs in mice, conventional DCs (cDC) and plasmacytoid DCs (pDC). cDCs are further subdivided into $CD8^+$ or $CD8^-$, with the former being mainly involved in cross-

[p] Dendritic cell: From Greek δένδρον "tree", due to their tree-like morphology. The dendrites of DCs are distinct from the dendrites of neurons.

presenting antigens to $CD8^+$ T cells via MHC Class I, while the latter present antigen via MHC Class II[131]. The final maturation stage the DCs reach is dependent on cytokine combinations and the niche which they occupy[132]. Cytokines important for DC differentiation and maturation are Flt3L and GM-CSF. After DCs capture and start processing the antigen, they mature and upregulate co-stimulatory molecules, such as CD40, CD80, CD83, CD86, and chemokine receptors, such as CCR7, to induce migration to the lymph nodes[133,134]. There, and at other secondary lymphoid organs like the spleen, DCs encounter T and B cells in specific areas called germinal centers. There they present the antigenic peptides on MHC molecules and if recognized, the cells of the adaptive immunity will be activated.

Apart from their role in antigen presentation to T cells, DCs can induce tolerance through induction of regulatory T cells (Tregs) or deletion of potentially autoreactive T cell clones[135]. They can also interact with other cells of the immune system, such as NK cells to promote anti-tumor effects[136,137], secrete pro-inflammatory cytokines[138], and prime B cell responses[139].

1.2.2 The adaptive immune system

The adaptive immune system appears later in the evolutionary tree and is found only in vertebrates. This is also evident by its vast complexity. The adaptive immune response starts days after an infection has begun and has to be primed by the innate immune system. The key to a proper adaptive immune response is antigen specificity and clonal selection of T and B cells that will eliminate the pathogen. Contrary to the innate immune system that employs cells of both myeloid and lymphoid lineages, adaptive immunity is exclusively mediated by lymphocytes. These originate in the thymus (T cells) or in the bone marrow (B cells) and have receptors that undergo gene rearrangements to recognize a specific antigen.

1.2.2.1 T cells

Up until the mid-1950s, the thymus, where T cells[E] develop, was considered to be a useless organ that had become redundant during evolution, since it was seen to atrophy during a person's teen years[140]. However, work mainly steered by Jacques Miller concluded that the thymus is an important organ for the induction of cellular, but also humoral, immunity[141]. During T cell development, hematopoietic progenitors migrate from the bone marrow to the thymus where they to generate a large population of immature T cell precursors or thymocytes[142]. In the thymus, T cells undergo a series of differentiation steps, which are

[E] T cells get their name from the thymus. The word thymus has uncertain origin; one speculation is that it is derived from its resemblance to the leaf of the plant *Thymus vulgaris* (thyme, Greek: θυμάρι). Alternatively, it comes from the Greek word θύμος, which translates to anger, heart, soul, desire, or life and has to do with its position in the chest above the heart, where emotions are felt, and ancient Greeks thought it might be the location of the soul.

typically defined based on the surface expression of CD4 and CD8, and happen at distinct microenvironments of the thymus[143,144]. Thymocytes first start as $CD4^{-}CD8^{-}$ double negative (DN) cells, then become $CD4^{+}CD8^{+}$ double positive (DP), and lastly mature into single positive (SP) $CD4^{+}$ or $CD8^{+}$ T cells[145]. The DN stage can be further subdivided into four distinct stages (DN1-4), based on their expression of CD44 and CD25[146]. The T cell receptor (TCR) is comprised of two chains, the α and β chains, and is constructed during the first two differentiation stages (DN2-3 and DP) via V,D, and J gene recombination[147].

$CD4^{+}$ T cells are generally referred to as helper T cells (Th) and $CD8^{+}$ T cells as cytotoxic T cells (Tc or CTL). Other T cell subsets are the T regulatory cells (Treg) and T cells that are part of the innate immune system, namely NKT, γδ T, and (mucosal associated invariant T) MAIT cells[148,149]. To ensure that T cells will be able to respond to alloantigens but not self-antigens, additional selection processes take place in the thymus, namely positive and negative selection. Positive selection takes place in the thymic cortex and ensures that DP T cells express a functional TCR capable of recognizing antigen peptide-MHC complexes on the surface of antigen-presenting cells (APCs)[150]. Self-antigens are expressed on MHC molecules by cortical thymic epithelial cells (cTECs). Only the thymocytes that interact with MHC Class I or MHC Class II appropriately (not too strongly or too weakly) will receive a survival signal. Thymocytes that do not fulfill this step die in a process called "death by neglect". This process ensures that the selected T cells have an affinity that fulfils useful functions. DP T cells that have interacted well with MHC class II molecules eventually become $CD4^{+}$ cells, whereas those that interact well with MHC class I molecules become $CD8^{+}$ cells[151]. When positive selection is completed, long-term survival of these T cells and migration into the medullar compartment of the thymus is ensured, where they undergo negative selection. While in the medulla, thymocytes are again presented with a self-antigen presented on the MHC complex of medullary thymic epithelial cells (mTECs)[152]. mTECs present all self-antigens present in the body through an enzyme called autoimmune regulator (AIRE), which turns on transcription of tissue-specific antigens that would not otherwise be present in the thymus. Thymocytes that interact too strongly with the self-antigen receive a signal that leads to apoptosis. Alternatively, some of these cells are selected to become Tregs. The remaining cells exit the thymus as immature, naïve T cells. This process is an important component of central tolerance and serves to prevent the formation of self-reactive T cells capable of inducing autoimmune diseases.

T cells become activated by interaction of their TCR with peptide-MHC complexes and their co-stimulatory receptors (CD28, ICOS etc.) with their ligands. TCR engagement is usually known as signal 1 and co-stimulation as signal 2. T cells need both signals to become activated, since TCR signaling alone leads to anergy. The TCR is a complex of several proteins; the TCRα and β chains as mentioned above, but also molecules called the CD3 proteins. These consist of CD3γ, δ, ε and ζ chains that arrange into CD3εγ and CD3εδ heterodimers and a CD3ζζ homodimer. The ζ chains contain a total of 6 ITAMs that can be phosphorylated and induce activating signaling[153]. Cytokines are also important for the proper activation of T cells and are referred to as signal 3. $CD4^{+}$ Th cells, for example, can

differentiate into multiple functional subsets, dependent on the cytokine milieu. IL-12 and IFNγ are critical cytokines for initiating the downstream signaling cascade to develop Th1 cells[154], which secrete mainly IFNγ and IL-2 and are involved in the elimination of intracellular pathogens. IL-4 and IL-2 are critical for Th2 differentiation, which mount immune responses against extracellular parasites and produce mainly IL-4, IL-5, IL-9, IL-13, IL-10, IL-25[155]. Other Th subsets include Th17, follicular helper T cells (Tfh), induced regulatory T cells (iTreg), the regulatory type 1 cells (Tr1), and the potentially distinct Th9 cells[155].

T cells also express an assortment of inhibitory receptors on their surface. These facilitate the adjustment of T cell responses to fit the inflammatory milieu in which they became activated. Inhibitory receptors can act to limit both co-stimulatory signaling and receptor ligation. For example, cytotoxic T lymphocyte antigen (CTLA)-4 is an inhibitory receptor that becomes upregulated on activated T cells. CTLA-4 yields intracellular phosphatase activity that dampens signaling downstream of the TCR and CD28 and it also acts as a competing receptor for CD80 and CD86, which it has a higher affinity for than CD28[156]. Other inhibitory receptors include programmed cell death-1 (PD-1) and lymphocyte activation gene 3 (LAG3)[157]. Blockade of these inhibitory receptors is being successfully explored clinically and aims to augment immune responses against various types of cancer[158,159]. This will be discussed more in subsequent paragraphs.

Ultimately, the goal of a T cell response is to develop life-long immunity through the generation of memory T cells. Historically, memory T cells were thought to belong either to the effector or to the central memory subtypes[160]. Central memory T cells express CD45RO, CCR7, L-selectin (CD62L), and have intermediate to high expression of CD44[160]. Effector memory T cells lack expression of CCR7 and CD62L, but have intermediate to high expression of CD44[160]. Effector memory T cells are mostly found in the periphery, while central memory T cells are found in the periphery and in lymph nodes[160]. Subsequently, numerous other populations of memory T cells have been discovered, such as tissue resident memory T cells that reside in tissues like the skin[161]. The single unifying characteristic of all memory T cell subtypes is that they are long-lived and can quickly expand upon repeated exposure to their cognate antigen.

1.1.2.2 B cells

The first functional indication of the existence of cells that we now know as B cells[F] came in the 1890s[162] when a publication highlighted the importance of circulating "antitoxins" in immunity against diphtheria and tetanus. These antitoxins were later shown to be

[F] B cells get their name, not from the bone marrow, but from the bursa of Fabricius, an organ similar to the thymus that is found in birds, where they were initially discovered and characterized.

antibodies[163] and the cells that produced them had pre-formed antibody receptors on their surface[164]. In 1965, Max Cooper and Robert Good published a landmark study that identified B cells as the mediators of the humoral[G] immune response. Working with chickens, they showed that cells that develop in the bursa of Fabricius (B cells) are responsible for antibody production and are distinct from the ones that develop in the thymus (T cells)[165].

In mammals, B cells derive from hematopoietic progenitors in the bone marrow. There, they develop from pro- to pre-B cells and thereafter to immature B cells. During these stages, they undergo V, D, and J gene recombination of the Ig heavy chain by enzymes RAG-1 and RAG-2, which will form the pre-B cell receptor (BCR)[166]. The same process happens to the V and J genes that form the Ig light chains κ or λ, which will associate with the pre-BCR to form the BCR[167]. B cells also undergo positive and negative selection while developing in the bone marrow. Positive selection occurs through antigen-independent signaling via both the pre-BCR and the BCR[168,169] and if these receptors do not bind to their ligand, B cells do not receive proper signals and cease to develop. Negative selection occurs through the binding of self-antigen with the BCR[170]. If it binds strongly to self-antigen, the B cell undergoes one of four fates: clonal deletion, receptor editing, anergy, or ignorance[170]. After the selection process, immature B cells migrate from the bone marrow to the spleen and pass through two transitional stages: Transitional (T)1 and T2[171]. Within the spleen, T1 B cells transition to T2 B cells[172]. T2 B cells differentiate further into either follicular (FO) B cells or marginal zone (MZ) B cells, depending on signals received through their BCR and other receptors[173]. Once differentiated, they are now considered mature or naïve B cells[172].

Antigens can activate B cells either with the help of T cells (T cell-dependent antigens) or without (T cell-independent antigens). T cell-dependent antigens cannot induce a humoral response in mice lacking T cells and generally lead to high-affinity antibodies. In T cell-dependent responses, the antigen is internalized through the BCR, degraded, and presented to T cells via MHC Class II molecules[174]. Following ligation of the TCR, T cells upregulate surface CD40L and secrete cytokines, such as IL-4[175]. CD40L binds to CD40 on B cells and acts as a co-stimulatory molecule for B cell activation. The cytokines also induce B cell proliferation, class switching, and affinity maturation by somatic hypermutation[175]. B cells then form a germinal center in a lymphoid follicle and then differentiate into long-lived plasma cells or memory B cells[176]. T cell-independent humoral responses can be induced in animals lacking T cells but the antibodies produced tend to have lower antigen affinity. Since B cells need additional signals to be activated, when they are not coming from the T cells, multiple receptors on the B cell have to be engaged. This can be mediated by PRRs like TLRs or by extensive cross-linking of the BCR. B cells from T cell-independent responses differentiate into short-lived plasma cells that produce antibodies mostly of the IgM class[177].

[G] Humoral immunity: From Latin: Humor "moisture", refers to the immunity found in bodily fluids not mediated by cells. Hippocratic medicine defined 4 humors (black and yellow bile, phlegm and blood), which could influence people's temperament and health. This is also present in the Indian Ayurveda system of medicine. Robin Fåhræus later concluded that the 4 humors were based on the observation of blood clotting.

1.2.3 Harnessing the immune system to treat cancer

Cancer immunotherapy is a field that employs the inherent ability of the immune system to fight cancer. The earliest report of immunotherapy for cancer treatment is from 2600 BC Egypt, where pharaoh Imhotep used a poultice, followed by an incision, to facilitate the development of an infection at the site and cause regression of the tumor[178]. More recently, in the 1890s, William Coley was the first to describe necrosis of the tumor induced by bacterial toxins. One of the proteins responsible for the induction of this process was later identified to be TNFα. Even more recently, in the late 1980s, cellular immunotherapy for cancer was introduced by Steven Rosenberg and colleagues. They reported tumor regression in patients with metastatic cancer who underwent different types of specific active immunotherapy[179].

Hematopoietic Stem Cell Transplantation (HSCT)

HSCT was originally attempted in 1957[180] and continues to be the only form of stem cell therapy that is widely available today for patients with hematological cancers. In HSCT patients undergo myeloablative conditioning and receive donor hematopoietic stem cells, which give rise to new immune cells that will, hopefully, fight off the cancer with better results than the host's own immune system had. This effect is known as the graft-vs-leukemia (GvL) effect. However, HSCT can come with the side effect of graft-vs-host disease (GvHD), in which the donor's immune cells start attacking the recipient's normal cells[181]. Efforts to further increase the success of HSCT by reducing rates of relapse, preventing and treating GvHD, reducing infectious complications, and increasing the availability of HSCT are constantly ongoing[182].

Cellular therapies

In the early days of HSCT, one method to refine the procedure and minimize GvHD was to put blood cells in culture with IL-2. This would generate the so called lymphokine activated killer (LAK) cells. When autologous LAK cells were transferred to patients with metastatic disease, in conjunction with IL-2 administration, they were able to efficiently kill tumor cells[183]. LAK cells are now known to consist mainly of NK cells[184]. Today, syngeneic and allogeneic NK cells are used for adoptive therapy of mainly hematological malignancies[185,186]. The process has been so refined that donor–host pair can be predicted by the expression of their KIRs, which is independent of the HLA-matching status[187]. T cells have also used in adoptive cell therapy, with some promising results in the treatment of solid tumors[188,189].

Cytokines

Cytokines are constantly used by immune cells as mediators of immune responses. The tumor microenvironment is generally an immunosuppressive milieu and the use of appropriate cytokines could potentially reverse it to a more proinflammatory state. IL-2 was one of the first cytokines used clinically to induce anti-tumor immunity and is approved for treatment of

malignant melanoma and renal cell carcinoma[190]. However, IL-2 is also stimulatory for regulatory T cells, which might counteract the beneficial effects of IL-2 in cancer treatment. Therefore, IL-15 that acts exclusively to activate NK cells has been effective in clinical use, also in combination with a potent IL-15 superagonist[191–194]. IFNα and other cytokines have also been explored.

Monoclonal antibodies

In 1987, CTLA-4 was identified by James Allison and colleagues[156] and was shown to inhibit T cell responses against tumor cells. Blocking antibodies against CTLA-4 were later shown to lift this inhibition from T cells allowing them to reject tumors *in vivo*[195]. Also in the early 1990s, Tasuku Honjo and colleagues discovered a molecule expressed in dying T cells, PD-1[196] and was shown to be another inhibitory receptor on T cells. Both CTLA-4 and PD-1 antibodies are now approved for clinical use with great success in hematological tumors[159,197]. Before these two, however, the first antibody treatment against cancer was anti-CD20 (Rituximab), which was approved by the FDA for treatment of follicular lymphoma in 1997. Rituximab targets CD20 on the surface of B cells and leads to death of the target cell. Therefore it is used in the treatment of B cell hematological malignancies[159,197]. Interestingly, the efficacy of many monoclonal antibodies have been shown to not only be dependent on T cell inhibition but also on the induction of NK cell-mediated ADCC due to engagement of the antibody Fc part with activating receptor CD16[198–200].

Cancer vaccination via DCs

As discussed in previous paragraphs, dendritic cells have many roles in controlling immune responses and in bridging innate and adaptive immunity. Therefore, DCs have for long been considered as optimal vaccines against cancer[201–208]. DC therapy invokes creating anti-tumor responses by triggering them to present tumor antigens to lymphocytes and thereby priming them to kill the tumor cells. One method of inducing DCs to present tumor antigens is by vaccination with autologous tumor lysates or antigenic peptides[209]. DCs can also be activated *in vivo* by expressing GM-CSF on tumor cells. This can be achieved by either genetically engineering tumor cells to produce GM-CSF or by infecting tumor cells with an oncolytic virus that expresses GM-CSF[210]. Another strategy is to remove DCs from the blood of a patient and activate them *ex vivo*. They can be activated in the presence of tumor antigens, which can be either a single tumor-specific peptide/protein or a tumor cell lysate, and are then infused back into the patient[209]. Sipuleucel-T is the only therapeutic DC vaccine against cancer currently in use and is approved for the treatment of prostate cancer[208,211]. The antigen used is prostatic acid phosphatase (PAP), which is present in 95% of prostate cancer cells, and it has shown significant increases in patient survival.

Receptor engineering

Adoptive transfer of tumor-infiltrating lymphocytes (TILs) has had some success, in for example, the treatment of malignant melanoma[188,189]. The limitation for T cell therapies lie in

the ability to isolate and expand high-affinity T cells clones that are restricted to tumor-associated antigens and in their limited *in vivo* expansion potential. However, by using gene transfer technologies, T cells can be genetically engineered to express a unique high-affinity TCR or a chimeric antigen receptor (CAR), both of which confer novel tumor antigen specificities[212]. Treatment with CAR-T cells has been successful in B cell malignancies, which display restricted expression of CD19[213]. Recently, CAR modifications for NK cells have also been explored[214,215], in autologous NK cells but also in NK cell lines, like NK-92[216,217]. CAR-NK cells currently under investigation are anti-CD19 for B cell malignancies and anti-EGFR for breast cancer and brain metastases[218].

1.2.4 Primary immunodeficiencies

Primary immunodeficiency diseases (PIDs) are a heterogeneous group of rare, chronic, genetic disorders that affect the immune system. The term has been expanded multiple times to include a wide spectrum of diseases[219]. The current 9 classes include combined T and B cell deficiencies, predominantly antibody deficiencies, congenital defects of phagocytes, genetic disorders of immune regulation, defects in innate immunity, autoinflammatory disorders, complement deficiencies, other well-defined immune deficiencies, and lastly phenocopies of PIDs[219]. According to the Immune Deficiency Foundation (IDF)[H], there are more than 300 classified PIDs.

All the aforementioned forms of PIDs are characterized by increased susceptibility to recurrent and/or severe infections, with the susceptibility to specific pathogens being dependent on the nature of the specific immune defect[220]. PIDs can arise from mutations in a single gene, generally arise early in life, and can affect one or multiple components of the immune system. SCID for example, which was mentioned in the prelude, can be caused by mutations in numerous genes, including *IL2RG*, *ADA*, *IL7R*, *JAK3*, *RAG1*, *RAG2*, *DCLRE1C* (*Artemis*), *TCRD*, *TCRE*, *TCRZ*, and *CD45*. What is also of interest is that in many PIDs one of the symptoms is autoimmunity. This potentially signifies that the threshold of activation is altered in immune cells from PID patients, not only to a higher threshold, which results in immunodeficiency, but also to a lower one. This could cause the cells to react less potently to real threats and at the same time mount a vigorous response to self, due to a breach in tolerance[221].

Of particular interest to this thesis are two immunodeficiencies caused by mutations in the Wiskott – Aldrich syndrome protein (WASp); the Wiskott – Aldrich syndrome and X-linked neutropenia. WASp is an important protein in the recruitment and remodeling of the actin cytoskeleton in response to receptor stimulation. These concepts will be further discussed in the second section.

[H] https://primaryimmune.org/

1.3 THE ACTIN CYTOSKELETON

Actin[I] is one of three filaments that comprise the cytoskeleton. The other two, intermediate filaments and microtubules, will not be discussed in the context of this thesis.

It is undeniable that cell movement is an integral part of many biological processes, including effective immune surveillance[222,223]. The actin cytoskeleton allows for the formation of specialized subcellular structures, such as lamellipodia, filopodia, stress fibers, and focal adhesions[223]. Therefore, abnormal regulation or activity of cytoskeletal components is often a cause of several diseases, including cancer[224], neurological disorders[225], and PIDs. Actin is one of the most abundant proteins on Earth[226] and is evolutionarily conserved in the eukaryotic cytoskeleton of organisms belonging to all taxonomic groups[227,228]. It has even been suggested that actin, tubulin, and histones have a common ancestral protein, based on their behavioral characteristics[229].

Actin exists in two forms; monomeric or globular (G-actin) and filamentous (F-actin). Regulating the homeostatic balance between these two actin forms ensures that cytoskeleton dynamics are carefully controlled and occur in response to extracellular stimuli[230–232]. The actin monomer is a 42 kDa protein that consists of four subdomains and is the basic unit for the double-stranded helical actin filament[233,234]. Actin is an ATPase[235] and ATP hydrolysis plays an essential role in the transition to F-actin (**Figure 1A**). Actin monomers bind ATP and ADP tightly, provided that Ca^{2+} or Mg^{2+} is present. One of these divalent cations associates with the β- and γ-phosphates of ATP, stabilizing its interaction with actin[233].

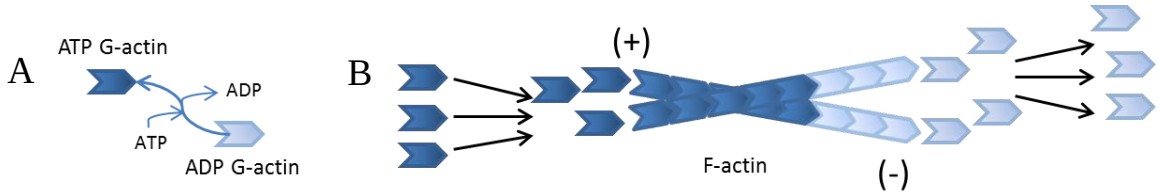

Figure 1: Monomeric and filamentous actin. **A.** Actin has ATPase activity and the conversion of G-actin from ATP- to ADP-bound and vice versa is a dynamic process in the cell. **B.** Actin treadmilling. The conversion of the ADP- to ATP-bound actin ensures the formation of actin filaments at the barbed (+) end of the filament. This occurs in a speedy manner. The conversion of the ATP- to the ADP-bound actin leads to the monomers separating from the filament at the pointed (-) end. This process is much slower.

The actin filament is produced with a process termed actin treadmilling, the kinetics of which are thermodynamically unfavorable[236] (**Figure 1B**). However, once oligomers are formed, spontaneous actin assembly can occur, since the ATPase activity of actin is enhanced upon polymerization[237]. This is provided that the concentration of actin monomer is above a critical concentration (0.1μM)[238]. During this process, actin monomers join the barbed (+) end of the filament in the ATP-bound state and disassociate from the filament preferentially at the pointed (−) end primarily in the ADP state (**Figure 1B**). More specifically, the newly

[I] The isolation and characterization of actin is commonly attributed to F.B. Straub in 1942 but it was first observed experimentally in 1887 by W.D. Halliburton who gave it the name "myosin ferment".

assembled part of the filament contains mostly ADP-Pi-bound actin with a cap of ATP-bound units at the tip, while older filaments have mainly ADP-bound actin. The ADP-Pi actin is structurally similar to ATP-actin[239] and both are more structurally stable that ADP-F-actin[240]. The release of phosphate triggers conformational rearrangements in the filament that result in less stable and more flexible ADP-actin filaments[241].

While nucleotide-dependent conformational changes control the structure and stability of the actin filament, there are additional control points that ensure proper regulation of actin polymerization. Numerous proteins like GTPases and G-and F-actin binding proteins (ABPs) play key roles in the precise manner actin filaments are formed. These will be discussed in the coming paragraphs.

1.3.1 Actin cytoskeleton structures

The polymerized actin filaments can further organize into different types of architectures: branched and crosslinked networks, parallel bundles, and anti-parallel contractile structures (**Figure 2**). These structures can act as mechanical elements to drive cell shape changes and motility. The crosslinked, parallel and antiparallel structures are briefly described below while the branched actin network will be discussed in subsequent paragraphs.

Crosslinked actin networks (**Figure 2, i and iii**) are involved in controlling the shape and mechanical integrity of the cell[242–246]. Proteins mediating actin filament crosslinking play no or little role during actin assembly. Instead, they connect existing polymerized actin filaments with each other, in order to generate a complex macroscopic structure[246–248]. Different crosslinking proteins provide different attributes to the actin filaments. For example, proteins that impose small crosslinking distances, such as fimbrin or fascin, pack the filaments close together into bundles[249,250]. Larger crosslinkers, such as filamin or α-actinin, are present in either bundles or networks, depending on their concentration[248,251–255]. Moreover, the rate at which actin networks are assembled can also influence the resulting structure. For instance, α-actinin preferentially generates long filaments instead of bundles when the rate of assembly is high[247]. These observations imply that there are multiple levels to control actin networks. Crosslinked actin networks, along with branched ones, are found in broad, sheet-like lamellipodial structures (**Figure 2, iii**). Lamellipodia are vital in providing the main driving force in two-dimensional cell motility[242].

Parallel actin networks (**Figure 2, iv**) are present in structures such as filopodia and microvilli[245,256,257]. As their name implies, parallel networks are made of actin filaments that are oriented with their barbed ends towards the same direction, usually facing the cell membrane, and result in finger-like structures. Fimbrin, fascin, and α-actinin are cross-linkers that make up these parallel networks and maintain the actin filaments in close proximity[258–260]. Two mechanisms of how bundle formation is initiated have been proposed, one requires the Arp2/3 complex and the other involves proteins like formins or Ena/VASp[256,261–271].

Antiparallel actin networks (**Figure 2, ii**) are important for cytokinesis and stress fibers that connect the cell cytoskeleton to the extracellular matrix via focal adhesion sites[272–278]. They contain the element of myosin-induced contraction. Crosslinking proteins, such as fimbrin and α-actinin, can also stabilize the actin bundles in the antiparallel configuration[274]. The interaction of myosin and antiparallel actin bundles is a two-step process that includes contraction and myosin-induced disassembly[264,279,280].

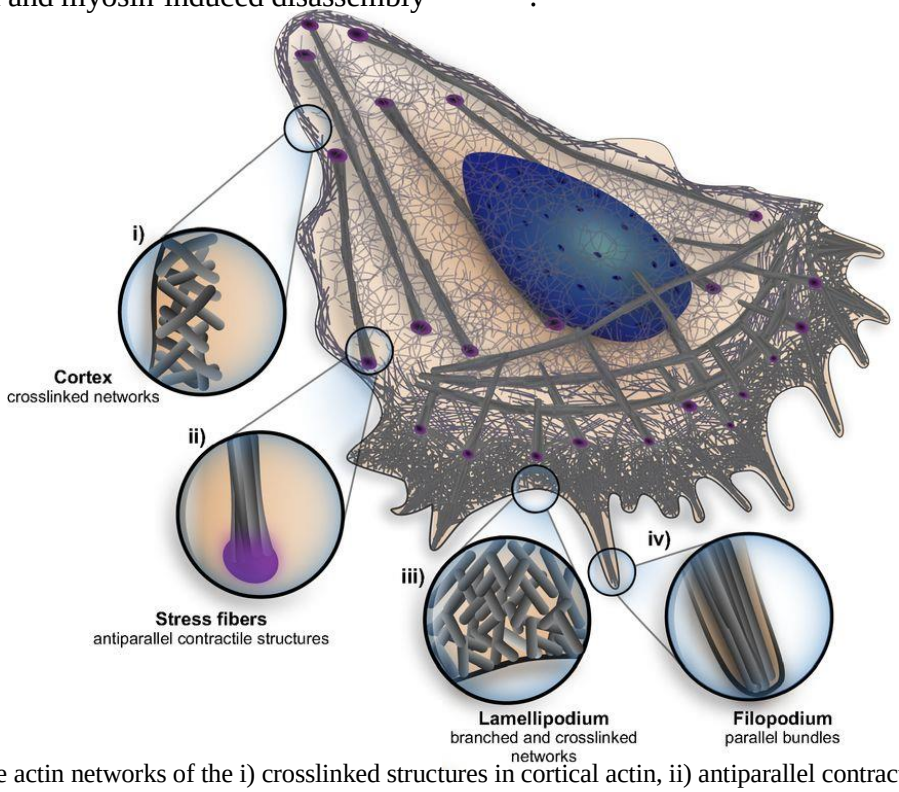

Figure 2: The actin networks of the i) crosslinked structures in cortical actin, ii) antiparallel contractile structures in stress fibers, iii) branched and crosslinked structures in lamellipodia and iv) parallel structures in filopodia. Figure modified from [281].

As important as it is to construct actin filaments and networks, it is equally important to disassemble them in a regulated fashion. Firstly, the distinction between disassembly and depolymerization should be made; depolymerization occurs in the filament ends[282] (**Figure 1B**), whereas disassembly essentially disrupts the mechanical integrity of the actin networks[283,284]. Cofilin can use fragmentation or severing to break down actin structures[285]. It can also bind to actin monomers and inhibit nucleotide exchange[286]. Myosin can also mediate filament severing. Actin bundles have been shown to slide along the myosin-coated surface at low myosin density, whereas they are disassembled at high myosin density[287].

1.3.2 Actin cytoskeleton regulators

The behavior of actin *in vivo* differs dramatically from that of purified actin *in vitro*. At the physiological actin concentration found in cells, over 99% would polymerize in mere seconds in a test tube. In contrast, approximately half of the total actin found in cells is not polymerized[226]. This difference can be attributed to actin binding proteins (ABPs) that regulate virtually all aspects of actin assembly. ABPs have different functions; some are

involved in severing and crosslinking, discussed above. Capping proteins, such as gelsolin and tensin, involved in *capping* of the barbed end to block the addition of new monomers[288,289], and tropomodulin, which acts exclusively on the pointed-end[290], all aim to decrease the overall length of the filament. The list of proteins that could be mentioned is endless, however, only three ABP groups will be discussed in more detail below, since they are of more relevance to this thesis.

Actin monomer-binding proteins. Proteins that bind actin monomers play a crucial role in controlling the pool of unpolymerized actin, by sequestering actin monomers and thereby modulating their addition to and dissociation from actin filaments. One example of actin monomer-binding proteins is profilin. It is an abundant protein that binds to the barbed end of an actin monomer and sterically inhibits elongation at pointed ends, but not at barbed ends[291]. Another actin monomer-binding protein is MKL1, which acts as a sensor of G-actin in the cytoplasm and will be analyzed further down.

Actin nucleation proteins. As mentioned above, nucleation of the actin filament is a thermodynamically unfavorable process and is additionally suppressed by proteins like profilin. Therefore, specific proteins are able to initiate actin polymerization in a regulated manner. One such protein is the Arp2/3 complex, which produces branched actin filaments. The actin-related proteins or Arps were created early during the evolution of eukaryotes, when the primordial actin gene was repeatedly duplicated and diversified into multiple protein families[292]. Arps share 17-52% sequence similarity with actin and are numbered Arp1–11, according to when they separated from actin. Arp2 and Arp3 are part of the Arp2/3 complex, along with five other subunits: ARPC1-5. The Arp2/3 complex is inactive but when it binds to the side of an actin filament, Arp2 and Arp3 move closer together and form a base for the growth of a branch[293]. The free barbed end of the new filament elongates, whereas the Arp2/3 complex anchors the pointed end of the filament firmly to the side of the existing filament, creating characteristic 70° actin-filament branches[294–296]. These observations have given rise to the dendritic nucleation model for actin-network. An alternative mechanism for branch formation has also been proposed[297], called the 'barbed-end nucleation' model, where the Arp2/3 complex binds to the free barbed end of a filament and two filaments subsequently grow from the branch[298]. Regardless of how they are made, actin filament branches are quite rigid and stable for tens of seconds. They can be further stabilized by proteins like cortactin[299] and cofilin[111].

Nucleation-promoting factors. A variety of proteins can activate the Arp2/3 complex to initiate actin polymerization and they all belong to the Wiskott – Aldrich syndrome protein family. This protein family currently consists of 16 members, including WASp and N-WASp, WAVE1-3, WASH, and newer members WHAMM and JMY[300,301]. All members share a conserved C-terminal domain (named VCA or WCA), required for Arp2/3 activation and F-actin nucleation. They also contain several unique N-terminal domains[302] that regulate their individual assembly into macromolecular complexes, subcellular localization and/or interaction regulator proteins. In this way, branched F-actin formation can be spatially and

temporally regulated throughout the cell and integrated downstream of various signaling pathways. There are also different regulatory mechanisms that govern the WASp family proteins. WASp and N-WASp are autoinhibited via intramolecular interactions and need activation by upstream proteins. WAVE proteins are instead constitutively active and their regulation downstream of Rac depends on the formation of protein complexes containing intermediary proteins. These differential modes of regulation are also reflected in the different biological activities of the proteins[303,304]. In that regard, WAVEs are generally required for the formation of lamellipodial protrusions and circular ruffles. Conversely, WASp and N-WASp are essential for the formation of specialized adhesive/invasive structures, termed podosomes or invadopodia, as well as being involved in the initial steps of clathrin-dependent internalization and in the movement of vesicles within a cell[302]. Additional weight will be given to nucleation-promoting factor WASp and actin monomer-binding protein MKL1.

1.3.3 WASp

WASp contains several domains including the Ena-VASP homology domain 1 (EVH1), also referred to as WASp homology domain (WH1), a basic region (B), a GTPase-binding domain (GBD), a proline-rich domain (PPPP), and a catalytic domain (VCA) also referred to as WCA domain (where the W stands for WASp homology domain 2 (WH2)) (**Figure 3**). The VCA domain is formed by three independent, structurally defined regions: the Verprolin homology domain, which binds to G-actin, the Central region, and the Acidic region, which mediates a direct interaction with the Arp2/3 complex[305]. All these domains allow for strict regulation of activity both by autoinhibition and external signals.

Structural studies demonstrate that under steady-state conditions WASp resides in an autoinhibited form, via binding of the GBD to the VCA domain (**Figure 3A**). This interaction prevents activation of the Arp2/3 complex[306]. WASp-interacting protein (WIP) associates with the EVH1 domain and stabilizes this autoinhibited configuration. WIP also protects WASp from degradation and facilitates the localization and maintenance of the cellular levels of WASp[307–310].

Rho family GTPases comprise a family of more than 20 members of small GTP-binding proteins that have key roles in rearrangement of the cytoskeleton, cell cycle progression, and vesicle transport[311]. The nucleotide-bound state of these GTPases is generally regulated by guanine nucleotide exchange factors (GEFs), which catalyze GDP to GTP exchange, and GTPase activating proteins (GAPs), which facilitate the hydrolysis of the bound GTP. Activation, by extracellular signals through various receptors, results in translocation to the plasma membrane thereby localizing their activity to discrete sites in the cell. The GTPase Cdc42 was the first protein shown to bind WASp and its interaction with the WASp GBD causes allosteric release of the C terminus, allowing binding of the Arp2/3 complex and actin nucleation (**Figure 3B**)[312–315].

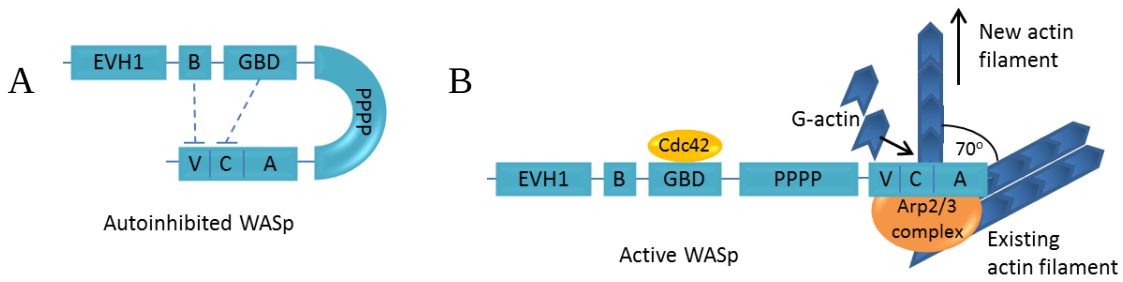

Figure 3: WASp in its inactive and active forms. **A.** The inactive form of WASp resides in an autoinhibited conformation. **B.** Upon receptor stimulation, WASp becomes activated by small GTPases and phosphorylation, which leads to its interaction with the Arp2/3 complex and the formation of branched actin filaments. Abbreviations: EVH1: Ena-VASP homology domain 1, B: Basic domain, GBD: GTPase-binding domain, PPPP: Polyproline domain, VCA: Verprolin homology/Central/Acidic domain.

Other signaling or posttranslational events can also modulate WASp function, apart from the Rho GTPases[316,317]. WASp autoinhibition is also modulated by phosphorylation of the GBD at tyrosine (Y)291[318,319]. In the autoinhibited structure, Y291 is inaccessible to kinases. However, upon Cdc42 binding, the GBD is opened, allowing for phosphorylation of Y291 by SH2-containing tyrosine kinases, such as Src. Phosphorylated WASp is able to promote Arp2/3-dependent actin nucleation *in vitro*, independent of Cdc42 binding. This suggests that phosphorylation converts WASp into a constitutively active form that may function to potentiate the duration and magnitude of its cellular signaling *in vivo*[318]. Consistent with this possibility is the observation that overexpression of a phosphomimic mutant of WASp, Y291E, induces filopodium formation in macrophages[320]. Phosphorylation at Y291 also promotes WASp ubiquitination at lysine (K)76 and K81 within the WH1 domain[321], providing a posttranslational mechanism to negatively regulate WASp *in vivo*. In addition to tyrosine phosphorylation, two serines (S), 483 and 484, which reside between the C and A domains, may also be subject to regulation via phosphorylation. Phosphorylation at these sites by casein kinase 2 induces a 7-fold increase in binding affinity between WASp and the Arp2/3 complex and significantly accelerates actin polymerization and nucleation *in vitro*[322].

1.3.3.1 WASp function in specific immune cells

In the *lymphoid lineages*, WASp is necessary for the survival and homeostasis of terminally differentiated lymphocytes, but does not seem to play a big role in the development of early progenitors[323–325].

T cells: $CD4^+$ T cell counts in WAS patients are usually within the normal to low range, but $CD8^+$ T cells are usually fewer[326,327]. Thymopoiesis is affected in WAS patients and WASp-deficient mice[324,325], with a reduced progression from double negative ($CD4^-CD8^-$) to double positive ($CD4^+CD8^+$) T cells found in mice. T cell receptor skewing has also been found in WAS patients[328] and WASp-deficient mice[329], with deviations from the diverse TCR repertoire taking place in older individuals. WASp-deficient T cells have been shown to be hyporesponsive after TCR-dependent activation[330–332], the cytotoxic activity of $CD8^+$ T cells

is impaired[333], and WASp-deficient Tregs fail to suppress effector T cells[334–337]. The latter might be an underlying cause for the autoimmunity observed in WAS[338,339]. Defective activity of follicular T (Tfh) cells residing in the spleen, which proliferate poorly, exhibit defective differentiation, and undergo increased apoptosis could also be a cause for the altered antibody production seen in WAS[340]. Mutations that lead to constitutively active WASp cause a skewing in the $CD4^+/CD8^+$ T cell ratio in XLN patients, due to increased numbers of activated ($HLA\text{-}DR^+$,$CD57^+$,$CD28^-$) $CD8^+$ T cells[341]. TCR-dependent activation of T cells in XLN induces a normal response[341,342], but T cells fail to adhere normally[342]. As briefly mentioned above, T cells exert their function by communicating with other immune cells via the immunological synapse (IS). Actin cytoskeleton remodeling and signal transduction has to occur for a proper IS to be formed. Upon ligation of the TCR, WASp is rapidly recruited to the IS to facilitate actin polarization. There it also mediates internalization of the TCR and sustains intracellular signaling, both of which do not occur in WASp-deficient T cells[330,343–350]. WASp-deficient T cells in mice also fail to polarize their cytokines towards the IS[351,352] and have reduced migratory responses, both *in vitro* and *in vivo*[353,354].

B cells: Absolute counts of circulating B cells in WAS patients are normal or slightly reduced and class switched memory B cells are normal in numbers[355]. WASp-deficient B cells display hyperproliferation, heightened antibody responses, and enhanced differentiation into class switched plasmablasts[356]. There is also an enrichment of autoreactive naïve B cell clones[357], which could contribute to the autoimmune phenotype of WAS patients. B cell receptor (BCR) signaling is impaired in WASp-deficient mice, with enhanced recruitment of negative regulators FcγRIIb and SHIP[358]. Memory B cells devoid of WASp exhibit impaired BCR and integrin signaling as well as chemotaxis in humans and mice[359]. WASp-deficient immature B cells, on the other hand, appear to have hightened BCR responsiveness, which promotes egress from the marginal zone of the spleen[358,360]. This could provide an explanation for the suboptimal T-independent antibody responses observed in WAS patients[356,359]. The number and function of B regulatory cells (Bregs) have recently also been shown to depend on WASp[361,362], so their dysfunction could also contribute to the autoimmune phenotype of WAS. Activating mutations in WASp results in failure of B cells to adhere, proliferate, and secrete antibodies in response to BCR stimulation[363]. There is also increased apoptosis associated with increased genomic instability, which could be a cause of the higher risk of hematological malignancies seen in XLN patients[363].

NK cells: WASp-mediated regulation of NK cell killing is a big part of this thesis and will be discussed in conjunction with the results obtained during experimentation. NK cell populations are often expanded in WAS patients[108]. However, NK cells with WASp deficiency demonstrate impaired actin polymerization and perforin accumulation at the NK cell-tumor cell IS, resulting in significantly reduced NK cell cytotoxic activity[108]. XLN patients have reduced NK cell counts[364], but not much is known about their function.

NKT cells: NKT cells are almost absent in WAS, but their numbers appear normal in XLT[365]. Mouse studies suggest that NKT cell maturation[366] but also function[365,366] is impaired in

WASp deficiency. Moreover, murine NKT cells devoid of WASp exhibit poor responses to glycopeptide antigens and fail to home to the sites of infection, instead they are retained in peripheral lymphoid tissues[365,366]. The role of NKT cells in XLN pathogenesis is poorly explored.

The role of WASp in the *myeloid lineages* seems to extend to the development of differentiated myelocytes. In XLN patients, bone marrow examination has revealed an intrinsic disturbance of myeloid differentiation, as maturation arrest was observed at the promyelocyte/myelocyte stage. Most defects due to WASp-deficiency in myeloid cells revolve around phagocytosis and podosome formation, both of which require vast actin remodeling. Myeloid cells from WAS patients exhibit impaired phagocytosis, since WASp has a role in forming the phagocytic cup[367–369]. In addition, monocytes, macrophages and dendritic cells from WASp-deficient humans and mice show almost completely abrogated assembly of podosomes[370,371].

Dendritic Cells (DCs):

DCs are important in priming T cell responses via the DC-T cell IS[372] and studies in humans and mice have shown that WASp-deficient DCs have a diminished T cell priming capacity[373–376]. DCs lacking WASp are also less capable of secreting pro-inflammatory cytokines[377,378] and of forming an IS with NK cells leading to lower NK cell responses[379,380]. Additionally, WASp deficiency leads to decreased DC migration to the skin resulting in a local potentiation of inflammatory T cells[381] and to increased antigen cross-presentation to $CD8^+$ over $CD4^+$ T cells[382]. Both of these mechanisms could contribute to the autoimmune skin pathology observed in WAS.

Neutrophils and other granulocytes:

Neutrophils are implicated in many immunodeficiencies, with neutropenia and recurrent bacterial infection being very common symptoms[383]. XLN leads to increased actin polymerization and increased cytoplasmic viscosity in neutrophil precursors, which affects mitosis and differentiation, and causes apoptosis in these cells ultimately leading to the clinical symptom of neutropenia[384]. WASp also plays important roles in neutrophil migration and adhesion, with both of those functions being diminished in murine models of WASp deficiency[385]. Mast cells that are devoid of WASp exhibit defects in granule exocytosis and cytokine production, with decreased capacity to respond upon FcεRI triggering[386].

1.3.3.2 WASp in cancer

A major factor controlling the metastatic nature of cancer cells is their motility. Alterations in the signaling pathways controlling the regulation of motility can lead to tumor cell invasion and metastasis. As mentioned previously, the driving force for membrane protrusion and

motility is localized polymerization of actin filaments at the leading edge of the cell. Recently, several studies revealed that molecules that link migratory signals to the actin cytoskeleton, including Rho GTPases and the WASp family members, are upregulated in invasive and metastatic cancer cells[222,224,387,388].

Podosomes[J] are conical, actin-rich, dynamic protrusions of the plasma membrane and have a polarized pattern of distribution in migrating cells, situated at the front border between the lamellipodium and lamellum[389,390]. They mediate cell contact with the extracellular matrix (ECM) and, importantly, induce ECM degradation at the contact sites. The formation of podosomes can be induced by oncogenic transformation of fibroblasts by *v-src*, suggesting the importance of these structures in oncogene-driven cell motility and invasion[391]. WASp is an essential component of podosomes in hematopoietic cells, such as macrophages and DCs. As already mentioned, DCs and macrophages derived from WAS patients, are unable to form podosomes[371,392], which suggests that WASp has an important role in podosome formation. Structures of this type are referred to as podosomes when they are found in normal cells and as invadopodia when they are found in cancer cells. However, they are proposed to have the same physiological function, which is remodeling of ECM structures. N-WASp has also been shown to be necessary for the invasion of several cancer cell lines and localizes at the invasion front[393–395].

1.3.4 The Wiskott - Aldrich syndrome

In 1937, German pediatrician Alfred Wiskott first described three brothers with a condition characterized by microthrombocytopenia[K], bloody diarrhea, eczema, episodes of fever, and recurrent ear infections. He concluded that these boys had a novel hereditary thrombopathy, since their sisters were not affected[396]. Almost 20 years later, American pediatrician Robert Aldrich reported a similar clinical phenotype in 16 out of 40 males, but not females, spanning over six generations of a single family he studied in 1954. This clearly pointed out the X-linked mode of inheritance of the disease[397]. The condition was termed Wiskott - Aldrich syndrome (WAS) after the two physicians who first described it. The gene mutated in WAS (*WAS*) was later identified on the X chromosome (position Xp11.22–p11.23) by positional cloning[398]. The protein product of the *WAS* gene is called the Wiskott - Aldrich syndrome protein (WASp) and it is ubiquitously expressed in non-erythroid hematopoietic cells. The family that was initially described by Wiskott was confirmed almost 70 years later to have a deletion of two nucleotides at positions 73–74 of *WAS*[399]. This resulted in a frameshift and premature termination of the protein[399].

[J] Podosomes: From Greek πόδι "foot" and σώμα “body, cell”. When researchers found these protrusions of the ventral membrane in *v-src* transformed fibroblasts, they considered these structures to be “cellular feet”.
[K] Microthrombocyte: From Greek μικρός "small", θρόμβος "lump" and κύτταρο "cell", refers to the platelet. Loss of platelets is what causes the common symptom of excessive bleeding in WAS patients.

WAS is a rare primary immunodeficiency caused by loss-of-function mutations in the *WAS* gene and 5 mutational hotspots in the gene have been identified by molecular analyses[400]. The incidence of WAS is estimated at between 1in10^5 and 1in10^6 cases per live births[401,402]. WAS makes up approximately 3% of all primary immunodeficiency disorders in the European Society for Immunodeficiency registry[326] and ranges from mild to severe, depending on how much of the protein expression is left[400]. Mutations that result in completely absent WASp cause classic WAS with a clinical phenotype of thrombocytopenia, eczema, recurrent infections, and an increased incidence of autoimmunity and malignancies[403,404]. Mutations in WASp that result in decreased protein expression cause X-linked thrombocytopenia (XLT), which is a milder disease characterized mainly by thrombocytopenia and occasionally milder eczema and immunodeficiency[405–407].

1.3.4.1 Tumorigenesis in WAS

Malignancy is a complication frequently observed in PIDs[408,409]. The tumor incidence in WAS patients is estimated to be 13-22% with a median age of onset of 9.5 years[403,410]. These malignancies are aggressive and patients generally have a poor prognosis. Most common are lymphoreticular tumors including non-Hodgkin's lymphoma (76% of the total tumors associated with WAS), both EBV and non-EBV driven[411], but also Hodgkin's lymphoma, Burkitt's lymphoma, myelodysplasia, and acute lymphoblastic leukemia[403,410,412–415]. Non-hematopoietic malignancies also occur with increased frequency[326]. $CD8^+$ T cells devoid of WASp are hyporesponsive and incapable of polarizing their granules, especially against tumor B cell lines[333]. This could contribute to a lower tumor immunosurveillance in patients.

1.3.5 X-linked Neutropenia

X-linked Neutropenia (XLN) is caused by gain-of-function mutations in *WAS*. The first mutation identified was the L270P mutation, which replaces the conserved leucine 270 into a proline in the GBD of the protein[341]. Since then, more mutations have been described, including the I294T and S272P mutations[364,416]. All mutations result in the GBD of WASp decreasing its affinity to the VCA domain, affecting the autoinhibited state of the protein. This leads to WASp being constitutively active and to increased actin polymerization, independent of Cdc42. Symptoms of XLN include recurrent major bacterial infections, severe neutropenia and monocytopenia, decreased NK and B cell counts, but low to normal platelets and normal serum IgA levels[417]. Bone marrow analysis demonstrates a hematopoietic arrest at the promyelocyte stage. Clinically, these patients remain well and are responsive to G-CSF therapy[326].

1.3.5.1 Tumorigenesis in XLN

XLN patients have an increased risk of developing cytogenetic changes indicative of chromosomal instability, myelodysplasia, or acute myeloid leukemia[418–421]. The link between constitutively active WASp and chromosomal instability is also supported in a murine model of XLN[363]. B and T cells from mice with the I296T mutation in WASp showed increased genomic instability, including chromosomal breaks, doublet and fused chromosomes, and tetraploidy. This could potentially be an underlying, intrinsic cause for the increased development of hematological malignancies in these patients.

1.3.6 Treatment of WASp-related immunodeficiencies

What follows is a review of the treatment options currently available for immunodeficiencies with loss-of-function mutations in WASp. However, these are also applicable to other PIDs.

Diagnosis: At the time of initial diagnosis, WASp protein expression by flow cytometry or Western blotting in peripheral blood cells is a rapid and effective screen for WAS[326,422,423]. Sanger sequencing of patient DNA is essential for confirming the diagnosis and should cover all exons and intron–exon boundaries, to identify possible splice site mutations[326]. Immune cell function and count tests can vary among patients and with time, but can be very important to assess an individual patient's progression. The combination of genetic, protein expression, and lymphocyte count data can be used to predict the prognosis of a WAS patient at presentation and to design the most effective treatment regime[326]. *Prophylaxis*: Patients are currently given antibiotics and immunoglobulin replacement treatment regardless of the test results. However, treatments of more specific symptoms, such as viral infections and autoimmune symptoms like eczema, are managed on an individual basis. *Hematopoietic Stem Cell Transplantation (HSCT)*: The first case of HSCT in the treatment of WAS was performed in 1978. It resulted in normal hematopoiesis and cure of hematological and immunological abnormalities[424]. Since then, practice has been refined by more conditional treatments prior to transplantation[326], with the donor stem cell source and the age at transplant being the main determinants of transplant outcome in WAS[326,425]. However, advances in the accuracy of HLA matching, improved supportive care and earlier routing to HSCT, have yielded a marked improvement in the outcome of HSCT and phenotype correction, with overall survival rates greater than 90%[326]. *Gene Therapy*: Gene therapy employs modified viral vectors that contain the normal *WAS* gene and *ex vivo* transduction of autologous hematopoietic stem cells. This approach completely eliminates the risk of alloreactivity and since there is a proliferative advantage of cells that have the normal gene expressed[426], there is a high success rate in reversal of the mutation. The first gene therapy study in WAS used a γ-retroviral vector[427]. This was unfortunately later shown in other diseases to cause retroviral insertion adjacent to oncogenes, resulting in leukemia in some of the patients[428]. Therefore, lentiviral vectors that cause far less insertional mutagenesis have since been implemented[429,430].

1.3.7 MKL1

The megakaryoblastic leukemia-1 (MKL1)[L] protein is a ubiquitously expressed transcriptional coactivator that transduces actin cytoskeleton rearrangements into gene expression via the transcription factor SRF. This ability of MKL1 is due to its sequestration in the cytoplasm where it binds directly to monomeric G- actin[431,432], via its conserved RPEL motifs (**Figure 4A**). Actin monomers occupy the N-terminal domain of MKL1 and block importins from gaining access to its nuclear localization signal and thereby impairing its entry to the nucleus[433]. When cytoplasmic G-actin levels decline, due to actin polymerization downstream of Rho GTPases, MKL1 translocates into the nucleus where it regulates transcription of cytoskeletal genes via the SRF[431,432] (**Figure 4B**). Recent evidence has also implicated nuclear actin in promoting MKL1 activation[434–437]. MKL1 binds to G-actin in the nucleus, and nuclear G-actin has been shown to facilitate nuclear export of MKL1 and to prevent MKL1 from activating SRF target genes[438].

MKL1 regulates many processes, including cardiovascular development[439], remodeling of neuronal networks in the developing and adult brain[434], megakaryocytic differentiation and migration[440], and many others. Despite the diversity in the functions of MKL1, the majority of MKL1 knockout mice are viable and fertile[439,441], possibly due to a functional redundancy between MKL1 and the other MRTF family members. Immunologically, MKL1-deficient mice have reduced platelet counts in peripheral blood and reduced ploidy in bone marrow megakaryocytes, suggesting a role for MKL1 in megakaryocyte differentiation and maturation[440]. They also show a similar phenotype to that of acute megakaryoblastic leukemia (AMKL) pathology associated with the chromosomal translocation t(1;22), in which the MKL1 and RNA-binding motif protein-15 (RBM15) genes are fused.

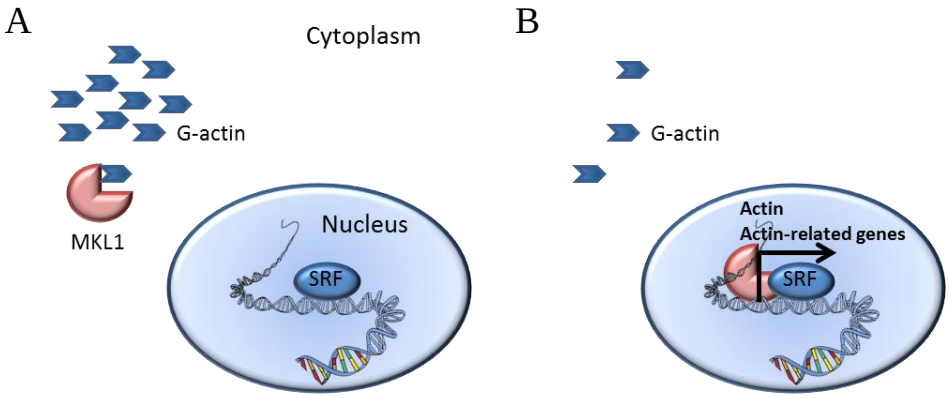

Figure 4: MKL1 mode of action. **A.** When actin is abundant in the cytoplasm, MKL1 binds G-actin, which hides its nuclear localization sequence. **B.** Initiation of signaling pathways, via among others the Rho GTPase, leads to the polymerization of actin, loss of MKL1 binding to G-actin, and translocation of MKL1 to the nucleus. There it interacts with SRF, which initiates transcription of actin and actin-related genes.

[L] MKL1 is also known with many other aliases, including Myocardin-Related Transcription Factor A (MRFF-A) and Megakaryocytic Acute Leukemia (MAL).

2 AIMS

The overarching aim of the present thesis was to investigate the impact of actin cytoskeleton protein regulators on mechanisms of tumorigenesis and the immune system in general.

The specific aims of the constituent papers were:

Paper I: To characterize NK cell function in a mouse model of WAS and to investigate the potential of IL-2 treatment on WASp deficient NK cells *in vivo*.

Paper II: To examine the NK and T cell function in mouse models and patients with activating mutations in WASp.

Paper III: To investigate the role of actin regulator MKL1 in the development of lymphoma.

Paper IV: To study the effects of a drug targeting a metabolic enzyme on the morphology and function of dendritic cells.

Paper V: To elucidate the function of WASp in the nucleus of T cells.

3 MATERIALS AND METHODS

All methodology used in this thesis is described in the individual papers. However, I would hereby like to mention a novel mouse model for molecular studies of WASp I aided in generating. The mouse model has yet to be featured in a paper, but I would like to bring forth its potential applications. We call it the **Triple-Tag WASp mouse**.

Many of the antibodies used for WASp have been shown to be cross-reactive to N-WASp since they are designed for parts of the proteins that are identical; WASp and N-WASp share over 50% sequence homology. We therefore took the approach to engineer a mouse with WASp connected to various protein tags. Three individual protein tags were added into exon 1 of WASp, immediately after the start codon (**Figure 5**). The protein tags were three Flag tags, one Myc tag and one Avitag. Flag and Myc are well-established protein tags with highly functioning antibodies commercially available. They are commonly used for assays like immunoprecipitation (IP) and co-immunoprecipitation (co-IP). Avitag is a protein that contains a very specific amino acid sequence that can be biotinylated at an exact lysine (K) contained within it by a bacterial ligase called BirA[442]. This can be used in conjunction with fluorescently labelled streptavidin or anti-biotin to perform high resolution localization studies of WASp.

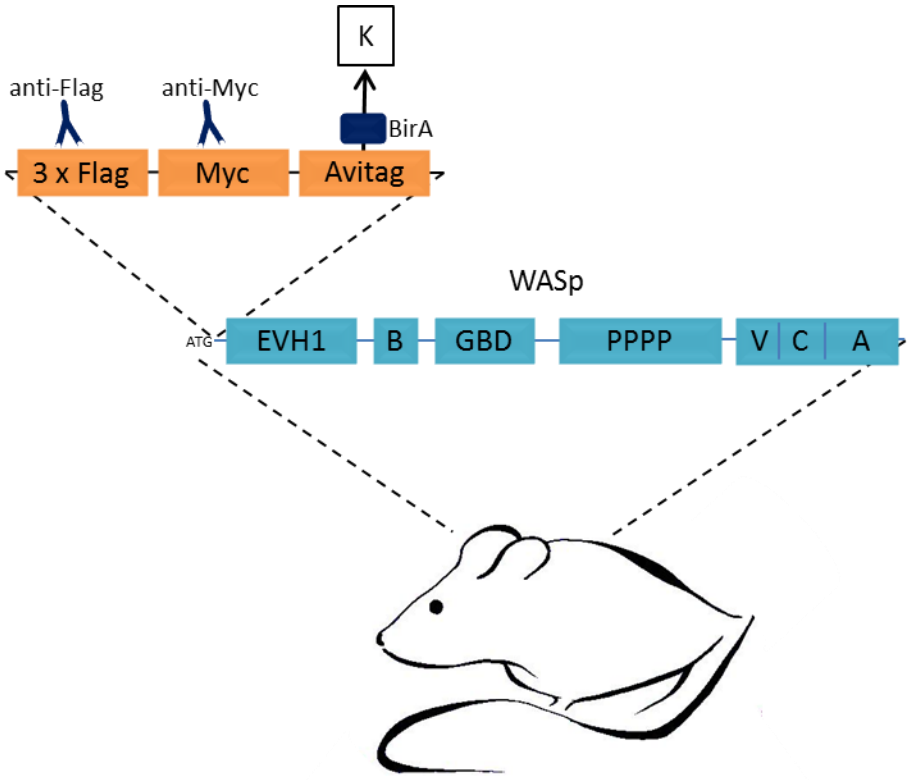

Figure 5: The genetics of the Triple-Tag WASp mouse. The three protein tags were added in exon 1 of WASp.

The Triple-Tag WASp mouse can also be crossed with mice engineered to express BirA, to obtain specific *in vivo* biotinylation of the Avitag *in vivo*. Imaging experiments of WASp localization in response to stimuli can then be conducted.

4 RESULTS AND DISCUSSION

4.1 WASP-DEFICIENT NK CELLS ARE CAPABLE OF RESPONDING TO TUMOR CELLS WHEN IL-2 IS PRESENT

In previous mouse studies, data on WASp-deficiency and tumorigenesis has been somewhat contradictory. When WASp-deficient (WASp KO) mice were crossed with tumor-prone mice that lack cyclin dependent kinase inhibitor 2a (WASp KO x Cdkn2a KO mice), they had an accelerated onset of tumor growth when compared to Cdkn2a KO mice[380]. Moreover, WASp-deficient mice have increased tumor growth of subcutaneously injected B16 melanoma cells and show increased metastatic nodes[380]. This is attributed in part to decreased NK cell-DC cross-talk in WASp-deficient mice[380]. Using two other models, injection of MTLn3 or MDA-MB-231 breast carcinoma cells into mammary glands, primary tumor growth was shown to be indistinguishable between wildtype (WT) and WASp-deficient mice[443]. Interestingly, the metastatic spread was decreased in WASp-deficient mice due to reduced capacity of tumor-associated macrophages to support cancer cell metastasis[443]. From these studies it is clear that the extent of how prone WASp-deficient mice are to increased tumorigenesis may depend on the tumor context.

In **paper I**, we utilized numerous lymphoma cells lines to assess tumor susceptibility in WASp-deficient mice, since most of the WAS patients develop hematological malignancies[403,410–415]. Using an *in vivo* imaging method we wanted to investigate the capacity of WASp-deficient mice to reject allogeneic tumor cells. We injected YAC-1 T cell lymphoma cells intravenously into Balb/c WT and WASp KO and monitored tumor cell rejection after a period of 5 hours (*Paper I, Figure 1A*). YAC-1 cells localize to the liver after intravenous injection and were located there in both WT and WASp KO mice during our *in vivo* imaging. However, YAC-1 tumor cell rejection was similar in both mice (*Paper I, Figure 1B*). This pointed out no defect in rejecting highly immunogenic allogeneic tumors in WASp-deficient mice. Next, we wanted to monitor tumor growth of the A20 B lymphoma cell line in WASp-deficient mice. We injected the A20 lymphoma cells subcutaneously into syngeneic Balb/c WASp-deficient and WT mice (*Paper I, Figure 6C*). We followed the mice with *in vivo* imaging over a period of 6 days, to capture early tumor growth. During this period, we saw no difference in the tumor growth of A20 cells in WASp KO and WT mice. From this we concluded that the susceptibility to syngeneic tumors was not altered in WASp-deficiency either. The third tumor model we used to dissect the outcome of WASp-deficiency in tumorigenesis and to address the specific role of NK cells was the RMA/RMA-S competitive assay(*Paper I, Figure 1C*)[18,444]. Both are T cell lymphoma lines, but RMA has normal expression of MHC Class I and RMA-S is MHC Class I-deficient[M]. RMA and RMA-S cells were labelled with different concentration of

[M] Both RMA and RMA-S cells were generated by mutagenesis of the parental lymphoma cell line RBL-5, but the RMA-S cell line underwent additional repeated selection for low MHC Class I. They were instrumental in the formulation of the "missing-self" hypothesis.

CFSE and injected intravenously into syngeneic C57Bl/6 WT or WASp KO mice. Preferential rejection via missing-self of the RMA-S tumor was assessed after two days in the spleen of the mice (*Paper I, Figure 1D*). We found no difference in the rejection capacity of NK cells against RMA-S tumor cells. To further investigate the *in vivo* functionality of WASp-deficient NK cells we performed the same competitive assay with differentially CFSE labelled WT and β2m-deficient splenocytes (*Paper I, Figure 1C*). When assessing the rejection capacity of WASp KO mice after two days in the spleen, we saw a marked defect in $\beta 2m^{-/-}$ cell clearance, compared to WT (*Paper I, Figure 1E*). This pointed to the fact that NK cells from WASp-deficient mice can respond to overwhelming activation signals from tumor cells, but cannot recognize lower activation signals coming, for example, from loss of MHC Class I on the surface of otherwise normal cells. NK cells from WASp-defient mice were also defective *in vitro*. They failed to degranulate and produce IFNγ in response to NK1.1 or NKp46 receptor stimulation (*Paper I, Figure 5A*). To test whether the *in vitro* and *in vivo* defects we observed were due to the phenotype of the WASp-deficient NK cells we assessed their development, maturation, and education status. The development of WASp-deficient NK cells in the bone marrow was normal since WASp-deficient mice had the same proportions of CLP, pre-NKP and NKP precursors as WT mice (*Paper I, Figure 2B*). Their maturation, based on CD11b and CD27, and their expression profile of inhibitory receptors were also indistinguishable from WT mice (*Paper I, Figure 2C and D*, respectively).

We additionally observed that pre-incubation of the NK cells with IL-2 could rescue the functional defect of the WASp-deficient NK cells *in vitro*. The NK cells could respond with degranulation and IFNγ to the same extent as WT after IL-2 treatment (*Paper I, Figure 5B*). Moreover, with IL-2 pre-treatment WASp-deficient NK cells were capable of polarizing their actin towards the activating surface (*Paper I, Figure 4E*). We therefore wondered whether IL-2 could rescue the *in vivo* defect of WASp-deficient NK cells to reject MHC Class I-negative splenocytes as well. After injection of IL-2 treated NK cells to mice that had previously received MHC Class I-negative splenocytes, WASp-deficient mice were capable of rejecting them to the same extent as WT. We finally asked whether the ability of WASp-deficient mice to successfully respond against tumors was mediated by IL-2. Indeed, there have been reports of IL-2, as well as other cytokines, being produced by various lymphomas and act as growth factors for the tumor cells[445–448]. Moreover, we see a correlation between IL-2 and increased survival in both neuroblastoma and diffuse large B cell lymphoma patients when using the R2 database (*Paper I, Figure 7E,F*, respectively). When assessing the lymphoma cells lines we used in our experiments they were all high in intracellular IL-2 expression *in vitro* (*Paper I, Figure 6A*) and *in vivo* (*Paper I, Figure 6B*), and could secrete the IL-2 (*Paper I, Figure Suppl.6B*).

To conclude, we show that IL-2 *in vivo* can modulate the responsiveness of WASp-deficient NK cells in the tumor microenvironment. B16 cells, that do not normally express IL-2 (*Paper I, Figure 6B*), have been shown to have an accelerated tumor growth and increased number of lung metastases in the absence of WASp[380]. Additional evidence for the role of IL-2 in the tumor microenvironment comes from observations that expressing IL-2 in B16 melanoma cells decreased tumor growth and long-term tumor control[449]. This local expression of IL-2 in the melanoma could increase the frequency of NK cells and

effector T cells at the tumor site and delay tumor growth, despite a simultaneous increase in infiltrating Foxp3^{+} Tregs[449]. More specifically for WAS, IL-2 has been shown to induce WAVE2 activation, which compensates for the absence of WASp downstream of IL-2R signaling[450]. Importantly, IL-2 has been used in clinical trials for prevention of cancer development in WAS patients, with promising results in restoring immune cells[451].

4.2 NK CELLS WITH ACTIVATING MUTATIONS IN WASP DISPAY SIGNS OF HYPERACTIVATION

Patients with XLN have mutations in *WAS* that lead to constitutive activation of WASp and a higher incidence of malignancies, as reviewed in paragraph 1.2.5.1[418–421]. Therefore, in **paper II** we wanted to test the rejection of tumors in our XLN mouse models, WASp^{L272P} and WASp^{I296T}. For this we made use of luciferase-expressing YAC-1 T cell lymphoma cells, injected subcutaneously, and *in vivo* imaging. The rejection of YAC-1 cells in normal mice is quite fast[452] so we imaged the mice over a period of two days. We observed that the rejection capacity of WASp^{L272P} mice was indistinguishable from WT (*Paper II, Figure 1A,B*). We additionally observed that WASp^{L272P} and WASp^{I296T} NK cells were still able to degranulate and produce IFNγ *in vitro* and in some cases did so to a higher extent than WT NK cells (*Paper II, Figure 1D,E*). We therefore wanted to test the *in vivo* functionality of NK cells in WASp^{L272P} and WASp^{I296T} mice. By using the *in vivo* rejection assay with MHC Class I-deficient splenocytes, we observed that after 48h, WASp^{L272P} mice were better than WT at rejecting them (*Paper II, Figure 1C*). There was also a marked difference in rejection capacity after 8h, with WASp^{L272P} showing increased rejection rates already then (*Paper II, Figure 1C*). This pointed out that NK cells from XLN mouse models were still capable of responding to stimuli and that they might have a lower threshold for activation. We excluded that the observed phenotype was due to a difference in WASp^{L272P} and WASp^{I296T} NK cell maturation or education, by assessing markers CD11b and CD27 and the inhibitory NK cell receptor repertoire (*Paper II, Figure 2B and 2C,D,* respectively).

4.3 ACTIN POLARIZATION TOWARDS THE IMMUNE SYNAPSE IS IMPAIRED IN WASP-DEFICIENCY AND DYSREGULATED WHEN WASP IS CONSTITUTIVELY ACTIVE

As discussed in the introduction, recruitment and remodeling of actin at the NK cell-target cell immune synapse is an important event in NK cell cytotoxicity[109,110]. NK cells from WAS patients cannot form functional synapses with tumor cells[108]. In accordance with those findings, we show in **paper I** that murine WASp-deficient NK cells fail to polarize their actin cytoskeleton towards tumor cells (*Paper I, Figure 4C*) or an activating surface (*Paper I, Figure 4D*). This is in conjunction with WASp-deficient NK cells being less able to localize their granules to the synapse as well (*Paper I, Figure 4C*). We therefore conclude that tumor cell killing is also impaired in WASp deficiency, when an NK cell-sensitive tumor that does not secrete IL-2 is assessed, as shown previously[380].

On the other hand, as we show in **paper II**, NK cells with activating mutations in WASp, WASp^{L272P} and WASp^{I296T}, are still capable of polarizing their actin towards the synapse with tumor cells (*Paper II, Figure 3B,C*). We observed the same thing by using high resolution imaging to capture the F-actin structures towards an activating surface, coated with antibodies against NKp46 (*Paper II, Figure 3D*). However, when looking at WASp^{L272P} and WASp^{I296T} NK cell actin polarization towards surfaces without any activating antibodies, we observed actin polarization there are well, whereas WT NK cells did not polarize their F-actin towards those surfaces (*Paper II, Figure 3D*). This spontaneous actin polarization towards a surface that does not contain any activating ligands seen in WASp^{L272P} and WASp^{I296T} NK cells also supports the notion of these mice having a hyperactive phenotype in their NK cells.

WASp and actin polarization in also important in the formation of T cell synapses and T cell-mediated cytotoxicity[333]. We therefore also assessed the ability of WASp^{L272P} and WASp^{I296T} CD4^{+} and CD8^{+} T cells to form synapses with antibody-coated beads. Both T cell subtypes from the XLN mouse models were capable of polarizing their actin towards the beads, whereas WASp-deficient T cells were not (*Paper II, Figure 5C-F*).

4.4 KLRG1 EXPRESSION IS INCREASED IN WASP-DEFICIENT AND DECREASED IN XLN NK CELLS

To test whether the *in vitro* and *in vivo* WASp-deficient NK cell defects we observed in **paper I** were due to their phenotype, we assessed the expression of numerous activating and inhibitory receptors on naïve NK cells. The defects could potentially either be due to decreased activation signaling or increased inhibitory signaling. We observed that WASp-deficient mice had higher KLRG1, CD69, DNAM-1, and LAG3 expression (*Paper I, Figure 3A,B*). KLRG1 and LAG3 are inhibitory receptors so increased expression of these two markers could cause the WASp-deficient NK cells to be hyporesponsive due to increased inhibitory signaling. DNAM-1 is an adhesion molecule that mediates activating signaling[57] and CD69 is a co-stimulatory molecule that contributes to sustain NK cell activation[453]. Therefore, the impact of the expression of the last two markers on WASp-deficient NK cells needs to be addressed with further experimentation. I would like to draw more attention to one of the inhibitory receptors, KLRG1.

The inhibitory role of KLRG1 has mainly been demonstrated in humans[454–456], where KLRG1 forms homodimers, something that proves essential for inhibitory function[79]. In experiments where KLRG1 is overexpressed in transgenic mice, NK cells could be inhibited by E-cadherin on K562 target cells but this was not observed at physiological KLRG1 levels. Hence, the inhibitory potential of KLRG1 in mice is rather weak and strong activation signals can override its inhibitory signal *in vivo*[79]. In their last CD11b/CD27 maturation stage, NK cells start expressing KLRG1 and differentiating into CD27^{-} CD11b^{+}KLRG1^{+} NK cells[457,458]. Therefore KLRG1 is considered as a marker of NK cell maturation. KLRG1 has also been associated with NK cell proliferation and regulation of NK cell homeostasis, since KLRG1^{+} NK cells accumulate and have a slower *in vivo* turnover than KLRG1^{-} NK cells[458]. Interestingly, the importance of the CD27^{low}KLRG1^{+}

NK cell subset for effective antitumor immunity was highlighted by adoptive transfer of this subset into T-bet-deficient mice and suppressing lung metastasis formation in a murine model of colorectal carcinoma[459]. Importantly, immunity to metastasis formation could also be restored by IL-15, which induced the development of $Eomes^{+}KLRG1^{+}$ NK cells from existing populations[459]. Therefore, it is possible that is was the $KLRG1^{+}$ NK cell population, which is increased in WASp-deficient mice, that mediated the anti-tumor response.

We also characterized the NK cells from mice with activating mutations in WASp in **paper II**, to see if the phenotype of hyperactivity we observed could be due to increased activation signaling and decreased inhibitory signaling. The only differential expression we observed in $WASp^{L272P}$ and $WASp^{I296T}$ mice, compared to WT, was that of KLRG1. Both XLN mouse models had lower expression of KLRG1 (*Paper II, Figure 2E*). Therefore, it is plausible that the NK cell hyperresponsiveness seen in $WASp^{L272P}$ and $WASp^{I296T}$ mice is caused, at least in part, by NK cells with lower KLRG1-mediated inhibitory signaling.

4.5 WAS PATIENT NK CELLS ARE HYPORESPONSIVE AND XLN PATIENT NK CELLS RESPOND NORMALLY TO STIMULATION

We showed in **paper I** that splenic WASp-deficient NK cells were incapable of responding to stimulation *in vitro* and peripheral NK cells from WAS patients have been shown to respond less to stimulation[108]. We therefore also wanted to assess the ability of splenic NK cells from a WAS patient to respond to PMA and Ionomycin stimulation. There was a marked decrease in the degranulation and IFNγ production of the WAS patient's NK cells, compared to healthy control (*Paper I, Figure 7C*). WAS patient NK cells had lower expression of CD56, leading to a large population of $CD56^{dim/negative}$ NK cells (*Paper I, Figure 7A*). The WAS patient's NK cells also had an increased expression of DNAM-1 and Granzyme B and lower expression of CD69 (*Paper I, Figure 7B*).

Conversely, when assessing the XLN patients' peripheral NK cells in **paper II**, we saw no major difference in the response of their NK cells to PMA and Ionomycin stimulation, compared to two healthy controls or their mother and sister (*Paper II, Figure Suppl.2B*). XLN patient NK cells had lower expression of KLRG1, much like their murine counterparts, and higher expression of Granzyme B (*Paper II, Figure 4C,D,* respectively). Interestingly, $CD4^{+}$ and $CD8^{+}$ T cells from the XLN patients had much higher expression of KLRG1 and higher Granzyme B content (*Paper II, Figure 6B,C,* respectively), but they could respond normally to PMA and Ionomycin stimulation. Their CD4/CD8 ratio was lower than in healthy controls, something that has been reported earlier[341]. Something new we observed in the XLN patients, however, was the appearance of a $CD4^{+}CD8^{low}$ T cell population, the significance of which should be further explored. Interestingly, this population has been shown to exhibit enhanced cytokine production, proliferation, and cytotoxic activity in viral infections, like HIV and CMV[460–462]. In conclusion, the phenotype and responsiveness of the NK cells from the XLN patients seems normal, however they exhibit a major loss of $CD56^{bright}$ and $CD56^{dim}$ NK cell populations in their peripheral blood (*Paper II, Figure 4A*), which could account for lower tumor surveillance.

Additionally, the responsiveness of their NK cells against K562 tumor cells is lower than the healthy controls' (*Paper II, Figure 4B*).

The discrepancy we saw between KLRG1 expression in the XLN patients and the murine models of XLN may have to do with the life span of humans and mice. The mice we used for our experiments are of young age, between 8 and 12 weeks. Comparatively, the patients we analyzed are in their late 20s. KLRG1 is associated with proliferation and KLRG1^{+} NK cells accumulate over time[458]. Since the age of analysis was so drastically different between the humans and mice, this could explain the difference in the KLRG1 phenotype.

4.6 MKL1 IS IMPLICATED IN THE DEVELOPMENT OF HODGKIN'S LYMPHOMA

The first thing I would like to bring up is that while MKL1 has been implicated in cancer, which will be discussed in detail underneath, complete MKL1 loss has been shown to lead to severe primary immunodeficiency[463]. A loss-of-function mutation in MKL1 found in a young girl caused reduced G- and F-actin content in lymphoid and myeloid cells, leading to widespread cytoskeletal dysfunction. MKL1-deficient neutrophils from the patient had a decreased phagocytic and migratory capacity and dendritic cells failed to spread and form podosomes.

MKL1, as the name suggests, was first discovered as part of a fusion protein (MKL1/RBM15) in acute megakaryoblastic leukemia (AMKL)[464,465]. However, it is not clear whether the tumorigenic properties of the MKL1/RBM15 translocation results from reduced expression and altered specificity of RBM15 and/or MKL1, or whether the fusion protein possesses oncogenic properties. MKL1 has been implicated in several malignancies apart from AMKL, most notably in breast[466,467] and liver cancer[467]. The function of MKL1 in breast cancer in coupled to the function of the estrogen receptor (ER). The Rho/MKL1 signaling pathway has been shown to be induced during the epithelial-mesenchymal transition. This results in an impaired ability of ER to efficiently transactivate estrogen-regulated target genes. MKL1 could therefore be implicated in the suppression of the protective role exerted by ER against tumor progression and metastasis[466]. Deleted in Liver Cancer 1 (DLC1) is a tumor suppressor protein that is lost in cases of both breast and liver cancer. DLC1 loss leads to the activation of the Rho/MKL1 pathway and to permanent MKL1 nuclear localization, which leads to constitutive activation of its target genes. MKL1 depletion in DLC1-defient cancer cells suppresses tumor cell migration, proliferation, and growth[467]. Recently, a deletion in MKL1 was found in two triplets who have developed Hodgkin's lymphoma (termed HL1 and HL2)[468]. The deletion is situated in the first intron of MKL1 (*Paper III, Figure 1B*) so it is not predicted to affect the translated protein sequence per se but it might affect the transcription and/or posttranscriptional modifications of the RNA, and thereby affect the expression or function of the protein. The third triplet that carries the mutation remains lymphoma-free (HL0) (*Paper III, Figure 1A*).

In **paper III**, we attempted to dissect the role of the MKL1 in the development of lymphoma. Hodgkin's lymphoma has many characteristics of an inflammatory/infectious

disease with symptoms, such as fever and lymphadenopathy[469], and has previously been associated with immunodeficiency[470]. Therefore, we subjected the triplets' peripheral blood mononuclear cells (PBMCs) to the Flow-cytometric Assay for Specific Cell-mediated Immune-response in Activated whole blood (FASCIA), in order to assess their cells' ability to respond to various stimuli (*Paper III, Table Suppl.1*). We found that their B cells responded well to most protein antigens, but displayed a decreased proliferation response to pokeweed mitogen.

We obtained blood samples from the triplets with the MKL1 deletion, as well as from two age-and sex-matched controls. We analyzed both primary B cells and monocytes form the patients and generated lymphoblastoid cell lines (LCLs) by incubating PBMCs with EBV-containing B95-8 cell supernatant[471]. Generating LCLs allowed us to perform additional *in vitro* experiments than allowed by primary cells. Interestingly, we were able to show that the MKL1 deletion led to an increased MKL1 expression in the triplets' primary B cells and monocytes, compared to the healthy controls, when assessed by flow cytometry (*Paper III, Figure 1D and Suppl.1C*). In the LCLs, we were able to detect higher MKL1 mRNA and protein levels only in HL0, but not in HL1 and HL2 (*Paper III, Figure 1C, E-H*). To investigate the functional outcome of altered MKL1 expression in the triplets, we examined the transcription of several MKL1-target genes by RT-qPCR, including *RAC1*, *TLN1*, *ITGB2*, *ACTB*, and *SRF*. Transcripts of all MKL1-target genes assessed were increased in HL0 (*Paper III, Figure 1I*). Since MKL1 is involved in actin regulation, we examined the contents of F- and G-actin in both primary cells and LCLs. F-actin measurement by flow cytometry in primary B cells revealed higher F-actin content in HL2 and in primary monocytes all triplet cells had higher F-actin content (*Paper III, Figure Suppl.1C*). Only HL0 had higher G-actin content (*Paper III, Figure 1K-M*). To summarize this part of the study, we were able to pinpoint the phenotype of altered MKL1 expression in HL0, with increases in G- and F-actin content and in the transcription of MKL1-target genes.

We next started to assess the LCLs obtained from the patients for signs of tumor transformation, including spreading, aggregation, proliferation, and genomic instability. Since actin is important for spreading responses in B cells, we examined the ability of the LCLs to spread on glass microscopy slides. HL0 and HL1 cells had an increased capacity to spread and to form long dendritic protrusions (*Paper III, Figure 1N-O, Suppl.2A*) and covered a larger adhesive area (*Paper III, Figure 1P-Q).* LCLs typically grow in clusters when in culture and their aggregation is dependent on adhesion molecules LFA-1 and ICAM-1. These are both induced by EBV transformation[472]. To quantify aggregation, we set up live cell imaging and measured clustering of the LCLs over a period of 2 hours. All LCLs apart from HL0 started forming aggregates right away. HL0 had a slower initiation of the aggregation process and ultimately formed smaller clusters than the rest of the LCLs (*Paper III, Figure 2A-B).* This aggregation defect in HL0 cells was probably due to lower expression of CD11a, a subunit of LFA-1, in HL0 LCLs (*Paper III, Figure 2C-E).* To address whether B cells from the triplets had altered proliferation compared to control B cells, primary cells were stimulated with anti-CD40 and IL-4 for 3 days and stained with the proliferation marker Ki-67. B cells from the triplets had higher Ki67 stained cells, indicative of their higher proliferative capacity (*Paper III, Figure 2G).* Conversely, in the LCLs the assessment of proliferation by means of [3H]-thymidine incorporation revealed

that HL0 and HL2 had higher DNA synthesis rates (*Paper III, Figure 2H)*. While performing Hoechst staining to address possible changes in the cell cycle of LCLs from the patients we observed, in accordance with the [3H]-thymidine experiments, that HL0 had more cells in the S and G2/M phase of the cell cycle (*Paper III, Figure 2I)*. Interestingly, there were more HL0 and HL1 LCLs that had more than 4n DNA content, compared to the rest of the LCLs (*Paper III, Figure 2J)*. This was excluded to be due to multinucleated, Reed-Sternberg-like cells (*Paper III, Figure 2K)*. Lastly, LCLs from HL0 and HL1 had a higher proportion of cells with more than 46 chromosomes (*Paper III, Figure 2L-M)*.

In summary, we herein present the first association of an MKL1 mutation with Hodgkin's lymphoma. The observed protrusions and increased spreading in HL0 LCLs could be associated with podosome/invadopodia formation. Decreased aggregation in the same LCLs, as well as increased proliferation and genomic instability could also imply a decline from the "normal" phenotype into a more mutational and metastatic state. The phenotype of cells obtained from HL1 and HL2 seems to fluctuate between the HL0 state and the healthy controls. On that note, it is of importance that the two triplets that previously developed lymphoma were treated in 1984 (HL1) and 2006 (HL2)[468] and that the third triple could potentially represent a pre-lymphoma state. The fact that he has not developed lymphoma to this day could depend on multiple reasons, including environmental, epigenetic etc. Additionally, using the R2 database[N] we present transcriptional data of heightened MKL1 expression in two types of lymphomas, compared to non-hematological cancers (*Paper III, Figure Suppl.3*).

4.7 INHIBITION OF POSODOME FORMATION IN DCS WITH A 15-LIPOXYGENASE-1 INHIBITOR

15-lipoxygenase-1 (15-LOX-1) is a vital enzyme that mediates the metabolism of polyunsaturated fatty acids. Specifically, it catalyzes the introduction of molecular oxygen O_2 as a peroxy residue into polyunsaturated fatty acids, especially arachidonic acid and linoleic acid. The expression of 15-LOX-1 is strictly regulated at the transcriptional, translational, and posttranslational levels[473] and its expression is tissue- and cell-restricted. The enzyme is predominantly expressed in airway epithelial cells, eosinophils, alveolar macrophages, dendritic cells, and reticulocytes[469]. 15-LOX-1 has been found to be implicated in anti-inflammation, membrane remodeling, and cancer development and metastasis. 15-LOX-1-deficient mice exhibit a prolonged inflammatory response, which implicates the enzyme in resolution of inflammation[474,475]. The enzyme has roles in suppressing inflammation in diseases like eczema, rheumatoid arthritis, asthma, cystic fibrosis, atherosclerosis, adipose tissue inflammation, insulin resistance, and Alzheimer's disease[474,475].

15-LOX-1 is also implicated in cancer and has been mapped in close proximity to the tumor-suppressor gene p53[476]. Loss of the p53 gene, or gain-of-function activities resulting

[N] r2.amc.nl/

from the expression of its mutant forms has been hypothesized to regulate the 15-LOX-1 promoter activity in humans and in mice, albeit in directionally opposite manners[476]. In colorectal, breast, and kidney cancer, 15-LOX-1 is downregulated and its levels sharply decline as the cancer progresses[475,477,478]. Mouse studies suggest that 15-LOX-1 might be a tumor suppressor[479]. However, the data there is conflicting as well[480]. In monocytes, the expression of 15-LOX-1 is stimulated with cytokines IL-4 and IL-13, but not IFNγ[481]. All this collectively suggests that 15-LOX-1 is tightly regulated to mediate proper inflammatory responses.

Hodgkin's lymphoma is considered a highly inflammatory type of cancer, with the Reed-Sternberg cells, characteristic cells found in Hodgkin's lymphoma, constituting approximately 1-5% of the cell population in the tissue, while the rest of the tumor consists of immune cells[482,483]. There is also immense cross-talk between the immune cells and the tumor cells in Hodgkin's lymphoma, which contributes to disease pathogenesis[484]. High expression of 15-LOX-1 has been detected in the Reed-Sternberg cells[485]. 15-LOX-1 has been suggested to operate through its products, like eoxins, and contribute to the development and/or morphology of Hodgkin's lymphoma[485]. For these reasons, inhibiting 15-LOX-1 could be a viable option for Hodgkin's disease therapy.

As mentioned in paragraph 1.2.3.3, podosomes, or invadopodia as they are mostly referred to in cancer cells, are membrane protrusions with an ECM degradation activity formed by highly invasive tumor cells[222,389]. In **paper IV**, we use atopic and contact dermatitis as models for inflammation in humans, to dissect the role of DCs and 15-LOX-1. Human monocytes were shown to express 15-LOX-1 and produce 15-LOX-1 metabolites upon differentiation to DCs in culture (*Paper IV, Figure Suppl.3*). Importantly, we show that by using a novel specific inhibitor of the activity of 15-LOX-1, a substantial reduction in DC spreading and podosome formation was observed *in vitro* (*Paper IV, Figure 3*). The antigen uptake and migration capacity of the DCs were also reduced when 15-LOX-1 was inhibited (*Paper IV, Figure 5A,B and C,D,* respectively). This marked decline in DC migration could potentially in turn lead to less priming of T cell responses in the lymphoid organs the DCs would migrate to. Additionally, the impairment of the antigen uptake and processing capacity of the DCs could also lead to reduced T cell responses. However, when using the 15-LOX-1 inhibitor or 15-LOX-1 shRNA in DCs we could only detect a minor effect on the T cell response (*Paper IV, Figure 4A,B*). Therefore, further refinement of the role 15-LOX-1 in DCs is needed for it to be pharmacologically targeted.

4.8 WASP RESIDES IN THE NUCLEUS AND IS INVOLVED IN THE REGULATION OF A T CELL-SPECIFIC TRANSCRIPTIONAL PROGRAM

The concept that actin resides not only in the cytoplasm but also in its nucleus is now an accepted fact. However, it was received with a lot of skepticism for over 30 years when first observed in the late 1970s[486,487]. Early, and perhaps the most convincing, reports of actin in the nucleus came from studies using *Xenopus laevis* oocytes[488,489]. These identified actin as a component of the nucleus based on its ability to polymerize, be decorated by myosin motor domains, and bind DNase I, all hallmarks of actin. Actin regulators like N-

WASp have also been found in the nucleus[490–494]. N-WASp is known to shuttle between the cytoplasm and the nucleus and is present in a large nuclear-protein complex within the nucleus[490]. This complex includes, among others, actin and RNA polymerase II and regulates gene transcription of target genes. The role of WASp in the nucleus is far less studied; WASp has only recently been found there and seems to directly mediate gene transcription[495–497].

T cell development, discussed in paragraph 1.1.2.1, heavily relies on the activity of the actin cytoskeleton. For example, the formation of immune synapses between thymocytes and antigen-presenting thymic stromal cells is essential for T cell differentiation and selection. Moreover, lineage commitment is controlled by specific transcription factors and those are in turn strictly regulated. T cell development is triggered by specific Notch signaling and the induction of transcription factors GATA3, TCF1, Bcl11b, and the E-proteins HEB (TCF12) and E2A[498,499].

WASp is important for T cell development and function (paragraph 1.2.3.1) and clinical symptoms of WAS include both immunodeficiency and autoimmunity. This can be considered a bizarre combination, with immune cells both not reacting to external pathogens while vigorously responding to self. This can also be interpreted as WAS patients exhibiting defective Th1 yet maintain functional Th2 immunity, which could be a sign of WASp activity directly in Th1 immunity. WASp has previously been described as a transcriptional regulator of TBX21, a Th1 master regulator gene[495]. This is based on the observation that WASp exists in the nucleus and is present at the proximal promoter locus of TBX21, but not GATA3 (a Th2 regulator) or RORγt (a Th17 regulator), as is F-actin itself. Additionally, WASp could associate with enzymes responsible for histone methylation at the TBX21 promoter during Th1 differentiation. In human T cells that lack WASp, TBX21 promoter dynamics are repressed, which is followed by lower T-bet expression and impaired Th1 cell functionality. Importantly, the observed inhibition of Th1 gene transcription and differentiation in the absence of WASp was ameliorated when WASp expression was restored in a patient-derived T cell line. Until now, the leading hypothesis for the molecular basis of WAS was the impairment of cytosolic actin polymerization. It is unclear how this causes a selective defect like the one observed in TBX21 gene expression and therefore suggests a role for nuclear WASp. Furthermore, murine T cells with overactive WASp and enhanced actin polymerization also have a defect in T-bet induction, which results in WAS-like immunodeficiency[500]. Another link between WASp and gene transcription was drawn by the use of a WASp mutant defective in actin polymerization. This mutant WASp was seen to potentiate NFAT transcription following TCR engagement[496]. Moreover, in *in vitro* derived Th1 cells, WASp interacts with a subset of SWItch/Sucrose Non-Fermentable (SWI/SNF) complexes to drive transcription of TBX12-induced genes[497].

Based on these studies, WASp residing in the nucleus is undeniably playing a role in WAS pathogenesis and its role should be further dissected, as should its protein and DNA binding partners. To identify the genetic binding positions of WASp on a global genomic scale, we took the approach in **paper V** to perform chromatin immunoprecipitation followed by subsequent sequencing of the obtained gene fragments (ChIP-Seq) in mice.

Firstly, by using co-immunoprecipitation (co-IP), we observed that WASp localizes to the nucleus in thymocytes (*Paper V, Figure 1A*), which again highlights its potential role in T cell development, and in splenic $CD4^+$ T cells (*Paper V, Figure 1B*), which speaks to the requirement of WASp throughout a T cell's life. Interestingly, WASp was almost exclusively detected in the thymic nuclear extract, compared to the nuclear extract of spleen $CD4^+$ T cells, which had equal amounts in the nucleus and cytoplasm. The significance of this remains to be uncovered. When performing the ChIP-Seq experiments, we identified 257 WASp-interacting genes in thymocytes and 455 WASp-interacting genes in splenic $CD4^+$ T cells (*Paper V, Figure 1C*). These datasets were obtained after exclusion of genes also found in antibody-free immunoprecipitations. When comparing thymic and splenic T cells, 15 common WASp-interacting genes were identified (*Paper V, Figure 1C*). WASp was found to cluster around transcriptional start sites (TSS) (*Paper V, Figure 1D*) and interacted with both intergenic and genic regions (*Paper V, Figure 1E*). Functionally, WASp was found to associate with RNA Polymerase II (RNA Pol II) genes (*Paper V, Figure 2A*), the functions of which can be categorized into 13 groups (*Paper V, Figure 1F*). These groups include for example transcription factors NFATC2 and TCF12. The WASp-enriched sequences of *TCF12*, among others, were additionally found to have active epigenetic marks of transcription (H3K4m3, H3K9a, H3K27a) and of active enhancers (H3K4m1) (*Paper V, Figure 2B*). Therefore, we conclude that WASp mediates T cell-specific transcription events.

To assess if this active transcription we observed really was WASp-dependent, we performed a transcriptional activity assay in T cells from WASp-deficient mice. This assay entails the incorporation of fluorine-conjugated UTP (FUrd) analogues into nascent mRNA molecules[501]. WASp KO thymocytes showed less intense FUrd-rich foci compared to those found in WT thymocytes, suggesting that WASp is required for basal transcription (*Paper V, Figure 4A-B*). To understand exactly how WASp impacts specific gene transcription, we compared gene expression of proteins involved in cytoskeletal and transcriptional networks between WT and WASp KO thymocytes with real-time qPCR. We found that many of them were differentially expressed (*Paper V, Figure 4C*), suggesting WASp transcriptional involvement in specific genetic loci. Of the genes we investigated, many of them showed higher and others lower expression in the absence of WASp. This suggests that WASp is involved in both transcriptional activation and inhibition (*Paper V, Figure 4D*). We also assessed basal transcription levels in thymocytes expressing one of the constitutively active mutations in WASp, $WASp^{I296T}$, and found similar intensity of FUrd-rich foci compared to WT thymocytes (*Paper V, Figure 4A-B*). This suggests that constitutively active WASp could still mediate transcription and does not alter the level of basal transcription. We also found that many genes were differentially expressed when WASp is constitutively active, with most of the genes tested showing higher expression in $WASp^{I296T}$ T cells (*Paper V, Figure 5B*).

TCF12 was one of the 15 WASp-enriched genes that was common between thymocytes and T cells from the spleen. We show that TCF12 expression is lower in WASp KO thymocytes, compared to WT thymocytes (*Paper V, Figure 6A*). Also, TCF12 had increased expression in $WASp^{I296T}$ and $WASp^{L272P}$ thymocytes, where WASp is constitutively active (*Paper V, Figure 6A*). We therefore conclude that the activation level

of WASp plays a big role in the subsequent expression level of TCF12 in the nucleus. To understand how the expression of these two proteins is connected, we checked WASp for any known binding sites of TCF12. Since we could not find any, by making use of gene ontology tools provided by MGI at NCBI Gene resource, we concluded that the interaction of WASp and TCF12 is indirect and perhaps mediated by a third party protein. Interestingly, we found that WASp-interacting genes were enriched for sequences identical to the binding sites of *TCF1* (*Paper V, Figure 6B*). Similarly, we identified several TCF1 binding sites in *TCF12* sequences (*Paper V, Figure 6C*). To test the hypothesis that WASp and TCF1 interact, we performed co-IP experiments that showed WASp and TCF1 interaction in nuclear extracts of thymocytes. Therefore, we conclude that nuclear WASp interacts with TCF1 to induce transcription of TCF1-target genes, which include TCF12 and TCF1 itself.

To summarize, **paper V** provides additional evidence on the existence of WASp in the nucleus and highlights its important role in the T cell-specific gene transcription machinery. TCF1 is one of the earliest transcription factors induced in T cell development[502] and is required to induce the pre-TCR complex in developing thymocytes[503]. Pre-TCR signaling ensures that proper T cell selection and development occurs. The fact that TCF1 expression is dependent on nuclear WASp is highlighted by lower TCF12 expression in WASp-deficient thymocytes. This could be part of the reason WAS patients and mice develop a skewed, autoimmune-prone T cell repertoire and that T cell responses via TCR stimulation as suboptimal. Moreover, we identify for the first time how nuclear WASp could be involved in the pathogenesis of XLN. Contrary to WASp KO thymocytes, $WASp^{I296T}$ and $WASp^{L272P}$ thymocytes had increased expression of TCF12.

4.9 THE TRIPLE-TAG WASP MOUSE MODEL

One concern when inserting a protein sequence into an already existing protein is whether that will be disruptive to the expression of that protein. The Triple-Tag WASp mouse was analyzed for its WASp expression and was found to retain WASp expression almost to the level of the wiltype (**Figure 6A**).

We then tested whether WASp could be specifically detected with the use of anti-biotin and anti-Flag antibodies (**Figure 6B**). Streptavidin and anti-Myc staining yielded similar results (data not shown). We cultured dendritic cells for one week in the presence of GM-CSF to obtain large cells with distinct actin structures to see if we can localize WASp to them. All antibodies tested showed similar staining patterns limited to the Triple-Tag WASp cells and not present in DCs from WT mice. The staining, initially expected to localize to the actin structures, had a more perinuclear distribution.

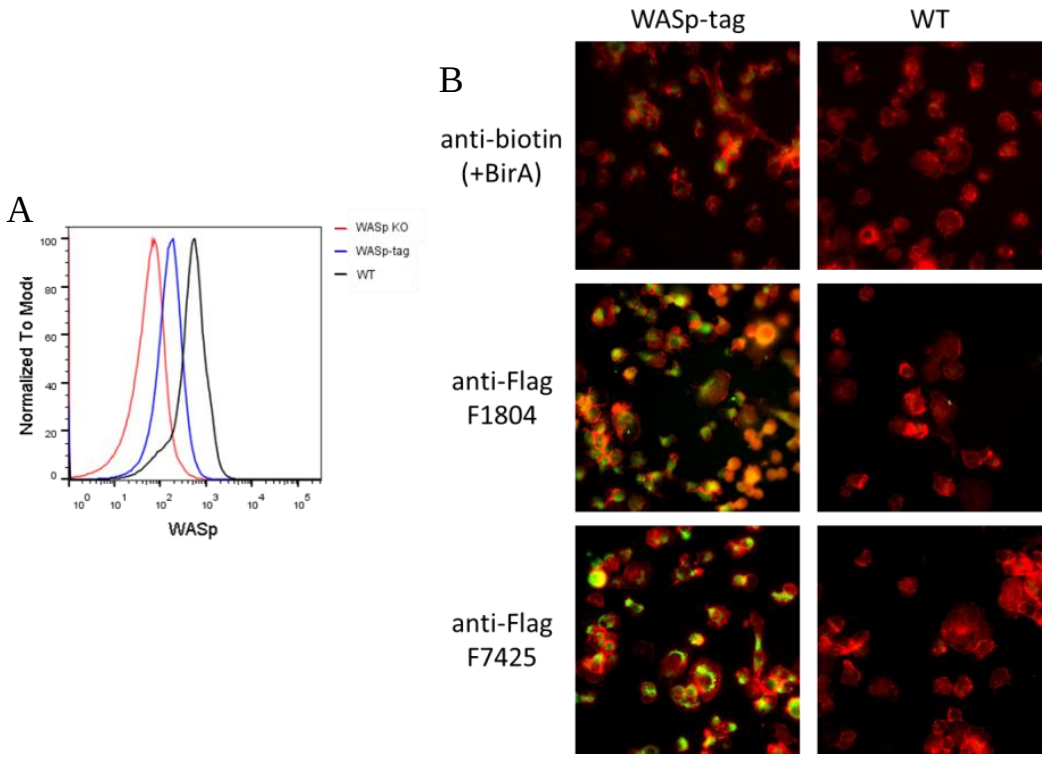

Figure 6: **A.** WASp expression in the Triple-Tag WASp mouse (WASp-tag) analyzed by intracellular staining for flow cytometry and compared to a WASp-defient (WASp KO) and a wildtype (WT) mouse. **B.** Dendritic cells from Triple-tag WASp (WASp-tag) and WT mice and intracellular staining of F-actin (phalloidin) in red and anti-biotin (top), anti-Flag clone F1804 (middle), and anti-Flag clone F17425 (bottom) in green.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

In my thesis, I have addressed the role of two actin cytoskeleton regulator proteins, WASp and MKL1, in the development of malignancy. Additionally, I have explored further roles of WASp in the nucleus and used an inhibitor to modulate an enzyme important for podosome formation.

The specific conclusions from each paper can be summarized as follows:

I. *WASp deficiency is detrimental for NK cell-mediated anti-tumor responses unless IL-2 is present.*

The role of IL-2 in the activation of NK cells has long been known. Interestingly, it seems to bypass signaling downstream of WASp and activates alternative pathways, fulfilling the need for NK cell activation in a proinflammatory, IL-2-containing environment. Therefore, our data is consistent with the notion that IL-2 is a vital component of the tumor microenvironment since overexpressing IL-2 in an otherwise IL-2 negative tumor could lead to the infiltration and activation of cytotoxic lymphocytes[449]. IL-2 has been shown to activate the WAVE2 pathway in humans, which is a WASp family member, and to initiate synapse formation in the absence of WASp[450]. The fact that WAS patients develop tumors due to their immunodeficiency is well-established. However, it is not known whether this is due to an immunosurveillance defect or whether their immune cells have intrinsic properties that make them tumorigenic since most of the tumors in WAS patients are hematological. The genetic instability in WASp-deficient cells has to our knowledge not been addressed. However, our data points more towards surveillance defects over intrinsic ones. The fact that patients can also develop non-hematological cancers, such as sarcomas, supports this notion. Additionally, the fact that we can rescue the WASp-deficiency defects, both *in vitro* and *in vivo*, points to a surveillance issue as well, which can be targeted by immunotherapy. Systemic administration of IL-2 was inefficient in restoring WASp-deficient NK cell function in our hands (data not shown), but further refinement of the protocol is necessary to draw conclusions of IL-2 immunotherapy of WASp-deficiency in mice. Importantly, IL-2 is being tested in clinical trials for the prevention of cancer development in WAS patients and seems to be well-tolerated[451].

II. *MKL1 is connected to lymphoma development*.

WASp overactivation compared to deficiency leads to a different set of symptoms in patients. However, the fact that both WASp absence and constitutive activation can have such detrimental effects speaks to the importance of actin cytoskeleton-related regulatory proteins. XLN patients also have a higher incidence of tumors, particularly hematological tumors of the myeloid compartment. This would argue for an intrinsic cause of tumor development. B and T cells have been shown to carry genomic instability and have high proliferation capabilities. Therefore, there may be cell cycle checkpoints that are inactivated in the constant activation status of WASp. This might be indicative of an additional role for WASp in the nucleus and investigating it could highlight potential mechanisms for tumorigenesis. NK cells and T cells when WASp is constitutively active seem to behave normally at an initial glance. However, in depth analysis of the synapse formation in NK cells from XLN mouse models revealed that they polarize their actin in the absence of activation signaling. Although seemingly trivial, this piece of data could indicate

hyperactivity in these cells and a lower threshold for activation. Since receptor expression analysis revealed no big differences between wildtype and the XLN mouse models, one hypothesis is that intracellular signaling is heightened and WASp is indeed hyperactive. An investigation of signaling pathway activation along with a clarification of the activation status of WASp would, therefore, be of interest. In order to elucidate if this seemingly hyperactive phenotype leads to increased functionality of XLN NK cells, additional functional experiments need to be performed as well. Of particular interest would be to evaluate if NK cells from XLN mouse models actually kill tumor cells and, if so, if they perform better than wildtype cells. Therefore, the use of live cell imaging would be of great value. We have crossed all our mouse models (i.e. one WASp-deficient and two XLN) to mice expressing GFP-tagged actin. This would allow for high resolution, real-time imaging of synapse formation and tumor cell death.

III. *MKL1 is connected to lymphoma development*.

We have shown that in a study of triplets, of which 2 have developed Hodgkin's lymphoma, MKL1 is overexpressed. This could be a potential link since MKL1 translocations and mutations have been seen in cancers previously. We also show that many lymphomas have a higher expression of MKL1. These data are indicative of the role of MKL1 in cancer development. However, to provide a direct link, further experimentation is necessary. The accumulation of genomic aberrations while maintaining proliferation is one indication of tumor transformation. Therefore, it would be interesting to perform additional stainings for genomic instability on the cells from the triplets, for example with γ-H2AX. One way to establish a direct link between mutations in MKL1 and lymphoma development would be to inject the LCLs into NOD-Scid-gamma (NSG) mice and monitor tumor growth and metastasis. These mice are immunocompromised and can therefore be used for human cancer cell inoculation without any rejection of the cells. By doing this and locating the differences in tumor growth and spread between cells with normal and cells with higher MKL1 expression, we can provide an *in vivo* link between MKL1 and lymphoma development.

IV. *Small inhibitors of 15-LOX-1 can inhibit podosome formation*.

When inhibiting the enzyme 15-LOX-1, we perturb podosome formation in DCs. While this is an interesting observation, we could not detect significant differences in T cell priming. Therefore, further investigation of molecules that can potently do that should be performed. Alternatively, the inhibitor we describe should be used in combination with additional drugs to provide multiple hits against immune disorders.

V. *WASp in the nucleus mediates speficic T cell transcription*.

Altered transcription due to WASp could potentially explain many of the symptoms in WAS and XLN patients. The use of mutant WASp constructs that do not mediate actin polymerization but maintain its role in transcription would elucidate the regulatory mechanisms necessary both these functions. The Triple-Tagged WASp mouse would be of great aid in the localization of WASp. Mass spectrometry of WASp would reveal important binding partners and shed light on pathways important for WASp function. That could also provide useful information for compensatory mechanisms employed in WAS and XLN.

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